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1994

PROGRESS REPORT

ON FOOD SAFETY RESEARCH

CONDUCTED BY ARS

Draft

EXECUTIVE SUMMARY

This report documents the ARS research progress in 1994 to assure the safety of food products of animal origin. Highlights of the achievements are outlined below. These are further documented in the report along with many others.

Food safety starts with a healthy animal also free of organisms which are pathogenic for humans. To detect all Salmonella in live animals with greater rapidity, accuracy and sensitivity, the basic information was obtained from which a test based on polymerase chain reactions (PCR) and recombinant proteins can be developed. Similar molecular techniques were utilized to develop tests that will identify E. coli pathogenic to swine and to cattle, including E. coli 0157:H7 .

Studies on colonization, growth and shedding of Salmonella in swine suggest that the respiratory route is important in establishing colonization of the GI tract. It was also demonstrated that isolation techniques during the weaning period are a practical method for raising and maintaining pigs free of Salmonella. Other research demonstrated that fecal shedding of E. coli 0157:H7 persists longer in young cattle than in adult cattle, and that the dose needed for shedding in adult cattle is very high (10^7). This pathogen also grew better in rumen contents from fasted animals than in rumen contents from well fed animals.

In addressing the ecology of pathogen reduction in the production environment, ARS demonstrated how Salmonella enteriditis (SE) grows and becomes virulent in mice which are a major reservoir of this pathogen for laying hens. Tests with a vaccine developed against SE in poultry demonstrated that immune modulation is a viable adjunct control strategy.

A defined culture of avian cecal bacteria was developed for use as a probiotic to control Salmonella colonization in poultry. Field trials were conducted to evaluate the efficacy of the characterzed culture against environmental salmonella in commercially raised broiler chickens. Salmonella isolations were decreased in both the skin feather samples collected from chickens in the rearing houses and in cecal contents sampled in the processing plants.

To control pathogens in the **slaughter and processing area,** ARS demonstrated that washing of trucks used to haul preslaughter swine could reduce confirmed levels of Salmonella to below detectable levels. Spraying of poultry carcasses with hot water rather than scalder immersion was shown to greatly improve their microbiological quality as well as use less water. The spray scalder will be installed in a processing plant for evaluation and optimization.

A modified microbial bioluminescence test that was shown to rapidly estimate the levels of generic bacteria on meat and poultry surfaces will provide a rapid indicator for process control in cattle, swine and poultry slaughter operations. It will be useful to both inspectors and industry personnel, and will make a vital contribution to slaughter/processing HACCP.

Post slaughter pathogen control comprises those studies addressing problems of product processing following the initial chilling of carcasses. A PCR test for Clostridium perfringes was developed which is far more rapid than traditional plate counts. Several flavor compounds and vegetable extracts that are already approved for use in food were found to inhibit many pathogens. The effectiveness of these compounds and extracts will be evaluated in actual food products.

Quantitative data on pathogen growth and survival were developed for E. coli 0157:H7, Listeria monocytogenes, Salmonella and Staphylococcus aureus using several different physical and chemical parameters. This quantitative data is the basis for models for pathogen growth and survival in food products. The models were incorporated into an easy-to-use program that runs on personal computers and were distributed to approximately 650 interested microbiologists in industry, regulatory agencies, and academia.

ARS demonstrated that irradiation at pasteurization doses can kill several strains of E. coli 0157:H7 in meat products, while keeping vitamin loss at acceptable levels. Further studies of the interaction of sodium chloride, nitrogen, temperature, etc., on irradiation of bacterial pathogens helped elucidate microbial physiology, and now contributes to the "chemiclearance" approach to use of irradiation in all meat products currently under regulatory consideration.

Residue detection focuses on drugs, environmental contaminants, and process generated contaminants. A salinomycin immunoassay with sensitivities in the low ppb range was developed and formatted as an ELISA for use with chicken liver. In its present format the assay does not require any complex sample preparation or depend on organic solvents.

Supercritical fluid extraction (SFE), both off-line and on-line, has been shown to be applicable to the analysis of volatile or semivolatile compounds in fire-damaged meat products. In most of the samples elevated levels of aldehydes such as hexanal or nonanal, have been found to be indicative of meat exposure to fire. A prototype Supercritical Fluid Extractor was assembled as part of a CRADA agreement between ARS and a company and was subjected to an extensive study to determine the instrumental specifications for the production model. It performed well in all tests conducted with tissue samples, and should meet the requirements needed in surveillance laboratories.

In initial studies which will lead to elucidation of the metabolism of dioxins in cattle, a radiolabelled 'non toxic' dioxin was administered orally to rats. Seventy seven percent of radiolabelled carbon was recovered in the feces and 17 per cent was recovered in the urine. Only one percent of the dose remained in the kidneys, liver, and carcass combined.

Methods for analysis of nutrients in food products is also included in this section of the report.

ANNUAL REPORT ON FOOD SAFETY RESEARCH CONDUCTED BY ARS

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CONTROL SALMONELLA IN DOMESTIC ANIMALS

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OBJECTIVE A:

To develop a cost effective means to prevent and/or control salmonellosis in poultry.

PROGRESS:

Efforts to develop defined cultures of avian cecal bacteria for use as probiotics to control *Salmonella* colonization in poultry were continued. Continuous-flow (chemostat) culture systems were utilized to select, isolate, and maintain a mixed culture of indigenous cecal bacteria that utilize glucose or other carbon sources to produce lactic acid and volatile fatty acids. Twenty nine bacterial isolates were isolated and identified in a mixed culture which is presently considered to be a characterized culture. The 29 isolates are composed of both facultative anaerobic and obligate anaerobic bacteria, some of which produce lactic acid and others which ferment lactic acid to produce volatile fatty acid end products. All isolates have been grown in pure culture and maintained as lyophilized cultures. Efforts are presently underway to recombine the pure culture isolates into a defined mixed culture using continuous-flow and batch culture systems.

Field trials to evaluate the efficacy of the characterized culture against environmental salmonellae exposure in commercially reared broiler chickens were conducted in March and April of 1994. A total of 30,000 chicks on three commercial farms were treated on the day of hatch and compared with equal numbers of non-treated control chickens on each farm. After six weeks of growout and prior to slaughter and processing, the number of salmonellae positive skin-feather samples collected from chickens in the rearing houses decreased from 21% (Control) to 8% (treated). In the processing plant, salmonella were isolated from the cecal contents of 5.7% of the control broilers and 2.7% of the treated broilers. Analyses of the results from each individual farm indicated that highly significant decreases ($P<0.01$) in salmonellae skin-feather and colonization occurred in the treated broilers on one farm while differences between control and treated chickens were not significant statistically on two of the farms. The efficiency of feed utilization improved in the treated chickens compared with the controls as indicated by a feed:gain ratio of 1.97 in the control flocks compared with 1.92 in the treated flocks.

One patent protecting the development and composition of the characterized culture has been issued and two other patents are pending. Exclusive licensing for commercial production of the culture has been completed through a CRADA with Milk Specialties Company, Dundee, IL.

Investigations to further define the roles of immune-mediated resistance and host inflammatory response on *S. enteritidis* (SE) translocation through the intestinal mucosal barrier and subsequent organ invasion have continued. Studies have demonstrated that intraperitoneal inoculation of leghorn chicks with immune cytokines produced by splenic T-lymphocytes of SE infected leghorn hens significantly increases the resistance of the chicks to SE organ invasion. Additional studies have verified that circulating heterophils infiltrate the sites of SE tissue invasion and actively participate in the host inflammatory response by phagocytosis and killing of SE organisms. Further studies have demonstrated that the immune cytokines actively influence heterophil migration and infiltration into sites of SE tissue invasion. The results strongly suggest that immune-mediated resistance and host inflammatory responses are important early defense mechanisms against SE tissue invasion and organ colonization. Recent studies have further demonstrated that *in ovo* injection of 18-day-old chick embryos with the immune lyphokine significantly increases resistance to SE organ invasion on the day of hatch.

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Droleskey, R.E., Oyofo, B.A., Hargis, B.M., Corrier, D.E. and DeLoach, J.R. 1994. Effect of mannose on *Salmonella typhimurium* mediated loss of mucosal epithelial integrity in cultured chick intestinal segments. Avian Dis. 38:275-281.

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Ha, S.D., Ricke, S.C., Nisbet, D.J., Corrier, D.E. and DeLoach, J.R. Serine utilization as a potential competition mechanism between *Salmonella* and a chicken cecal bacterium. J. Food Protect. (Accepted for publication).

PATHOGENESIS OF SALMONELLA ENTERITIDIS IN CHICKENS

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OBJECTIVE A:

Develop and evaluate the effectiveness of killed vaccines for protecting chickens against infection with *Salmonella enteritidis*.

Progress A:

Many recent reports have associated human illness due to *Salmonella enteritidis* (SE) with the consumption of contaminated eggs, considerable interest has been focused on the possibility of diminishing the incidence of SE infection in flocks of laying chickens by vaccination. Any reduction in intestinal colonization by SE or fecal shedding of SE associated with vaccination could have significant epidemiological significance. For example, reducing the incidence of intestinal colonization should also reduce the likelihood that subsequent events in the infection process (invasion, dissemination, production of contaminated eggs) will occur. Moreover, reducing the shedding of SE into the environment should reduce opportunities for infection to spread to other chickens. We have evaluated the protective efficacy of inactivated SE vaccine preparations (bacterins). After laying hens were vaccinated with the bacterins, they were later challenged with live SE to determine whether the vaccines would reduce the ability of the bacteria to colonize the intestinal tract and be shed in the feces. Although both the incidence of intestinal colonization by SE and the numbers of SE cells shed in the feces were significantly reduced by bacterin administration, many vaccinated hens still showed evidence of intestinal colonization (and some still shed large numbers of SE in their feces). The partial protection associated with vaccination in this study demonstrated to poultry producers and regulatory officials that vaccination could reduce the susceptibility of chickens to SE and, if applied together with other flock sanitation and infection monitoring strategies, could likely help reduce the horizontal transmission of SE within and between houses.

OBJECTIVE B:

Develop and evaluate sensitive and efficient methods for testing chickens and eggs for *Salmonella enteritidis*.

PROGRESS B:

We demonstrated that iron supplementation of incubating egg contents pools improved the recovery of *Salmonella enteritidis* (SE) by a direct plating culture method. Culturing egg contents for contamination by SE has become a common feature in proposed programs to control SE in laying flocks. Many such programs have been based on a direct plating culture method, which is rapid and comparatively inexpensive, although previous studies have suggested that it may be less sensitive than other methods. Efficient detection of small numbers of SE in experimentally inoculated egg pools has been shown to be heavily dependent on the expansion of the SE population during pool incubation, and requires about 10,000 times more SE cells than can be detected by broth enrichment culturing. We evaluated whether adding an iron source to incubating eggs pools would affect the growth of SE in those pools and the corresponding likelihood that SE in the pools would be detected by direct
plating. Iron supplementation led to a significant increase in the numbers of SE in egg pools after incubation and accordingly supported a significant improvement in the ability of direct plating to detect contamination. These studies demonstrated to regulatory officials and diagnostic laboratories that increasing the amount of iron available to support bacterial growth in incubating egg pools could increase the probability that a small number of SE cells would grow quickly to levels likely to be detected by direct plating.

OBJECTIVE C:

Investigate mechanisms of *Salmonella enteritidis* spread.

Progress C:

Fasting older hens to achieve a second egg lay increases the severity of an infection by *Salmonella enteritidis* (SE) and this occurs rapidly after the hens become infected. By 24 hours postinfection, more SE can be recovered from the intestinal tract and feces of fasted hens compared with unfasted hens and there is also more intestinal damage observed at this time in these birds. By 48 hours, more SE can be recovered from livers and spleens of fasted hens indicating that fasting also rapidly affects the spread of the organism throughout the body of the bird. Fasted hens are more susceptible to an infection by SE and this factor, coupled with the large amounts of the organism released by fasted infected hens, increases the transmission of SE within a group of birds. In experiments where a single hen among a group of hens was infected with SE, the organism spread rapidly through the fasted hens compared with little spread in the unfasted hens. By day 3 postinfection, 30% of the fasted hens were infected compared with 4.5% of unfasted and by day 10, 81% of the fasted hens were infected compared again with 4.5% unfasted hens. These results indicate that fasting can rapidly change the SE status of a flock.

OBJECTIVE D:

Investigate components of the chicken immune system and *Salmonella enteritidis* infection.

PROGRESS D:

Salmonella enteriditis infection and cytokine production. Tumor necrosis factor (TNF), an inflammatory cytokine produced primarily by macrophages, is elevated in the intestinal tract of *Salmonella enteritidis*-infected chickens. TNF activity in the intestinal tract was maximal at 24 hr postinfection and again at 8-10 days postinfection. This elevation was especially pronounced in the fasted, infected hens, a probable reflection of the increased intestinal inflammation observed previously. The role of this protein in the disease state is currently under investigation.

OBJECTIVE E:

Develop a virulence assay for assaying the population dynamics of *Salmonella enteritidis*.

PROGRESS E:

Detection of swarming by *Salmonella enteritidis* (SE). It was determined that there is predictable variation in the expression of key virulence factors on the outer surface of field isolates of SE that correlate with pathogenicity in chickens. Because of the variable expression of these molecules, it was possible to grow colonies that had distinguishing features which correlated with pathogenicity, thus resulting in the ability to quantify virulent variants in the hen house environment. We identified environmental selection pressures and stresses that induced variability so that investigations into the population dynamics of SE avirulent and virulent field isolates could be initiated. One of the more striking induced colonial variants displayed swarming, which is a type of multicellular behavior known to be associated with heightened virulence of bacterial pathogens. The ability to quantitatively assess the population dynamics of SE is expected to impact vaccine design since wide spread vaccination of densely stocked animals could be ineffectual if avirulent isolates are supplanted in the environment by virulent isolates which are able to evade immune responses. It appears that in addition to vaccination, maintenance of avirulent bacterial populations in animal environments or monitoring for the rapid expansion of virulent populations could be other useful methods to decrease food-borne outbreaks in humans.

OBJECTIVE F:

Assess the role of mice as reservoirs for *Salmonella enteritidis*.

Progress F:

Mice are a significant reservoir of invasive *Salmonella enteritidis* (SE). An 18 month assessment of mice captured in hen houses determined that field mice are a significant reservoir for invasive SE. During this period, 26% of mouse spleens were culture positive, while percent positive spleens ranged from 1 to 80% per batch. Serotype B *Salmonella*, presumably *S. typhimurium*, was the next most common serotype, but it was recovered from only 1% of spleens. Statistical analysis showed that SE positive spleens were more likely to be recovered from positive houses, although positive spleens were recovered from environmentally negative houses. Conversely, environmentally positive houses could be found even though there was no invasive SE found in captured mice. These findings suggest that environmental testing fails to detect SE in some cases, while inappropriately suggesting that all positive SE houses are hazardous. Swarming bacterial behavior, which is a type of accelerated growth associated with hypermotility and increased virulence, was seen in 4% of isolates. Quantitative assessment of lipopolysaccharide (LPS) structure indicated that swarming SE isolates were characterized by production of an unprecedented amount of O-antigen, which is the immunodominant region of LPS. These findings reiterate the central role of LPS structure as an indicator of virulence. Statistical analysis for differentiating populations showed that an experimental chick assay model using isolates with characterized O-antigen profiles produced the same curvo-linear profile as that derived from mouse data. These results indicate that the dynamics of infection in chickens and mice are probably similar, and that a published experimental infection model is an indicator of what happens in hen houses when mice or chickens are infected. Best curve fit analysis of ranked batch data indicated that recovery of positive spleens was at first logarithmic, then stationary, and finally exponential in hen houses. Swarming behavior was observed at the junction of stationary and exponential phases.

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CONTROL OF SALMONELLA IN DOMESTIC ANIMALS

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Completion Date: September 30, 1995

OBJECTIVE A:

Develop control procedures to prevent infection of eggs and chicks by salmonellae in hatcheries including treatment of equipment, eggs, chicks, and/or breeder flocks with chemicals.

PROGRESS A:

The use of vacuum with chemical disinfectants to eliminate artificially inoculated *Salmonella heidelberg* and *S. typhimurium* from hatching eggs has been studied. H_2O_2 (1.4%) under vacuum produced 68% more negative eggs as compared to H_2O_2 alone. With polyhexamethylene biguanide hydrochloride (PHMB) (0.035%) under vacuum produced 36% more negative eggs as compared to PHMB alone. The use of vacuum drew more of the chemicals into the egg to kill *Salmonella* without adversely affecting hatchability of the fertile hatching eggs. The effect of ultra-violet light to kill *Salmonella* on the surface of hatching eggs is being evaluated. In inoculation studies, a 10 minute treatment with UV light reduced *Salmonella* positive eggs from 100% (+) to an average of 30% (+). Optimization of this work is continuing.

The effect of ozone gas, UV light and microaerosoled H_2O_2 on bacteria levels and cross-contamination of *Salmonella* were tested in the hatching cabinets. Compared to controls, all treatments reduced aerobic bacteria, enterobacteriaceae, and *Salmonella* in hatching cabinet air samples. Aerosolized H_2O_2 was the most effective treatment to reduce bacterial and *Salmonella* cross-contamination in the hatching cabinet and in chicks grown for 7 days after removal from hatching cabinets. None of the treatments adversely affected hatchability or early chick morbidity or mortality. A CRADA has been initiated with Crystal Rivers Technologies to study the effect of ozone on the control of *Salmonellae* in the hatching cabinet.

OBJECTIVE B:

Develop control procedures to prevent infection of poultry by competitive exclusion cultures.

PROGRESS B:

A commercial company is negotiating with ARS for the exclusive right to market the patent pending mucosal competitive exclusion (MCE) product developed at Russell Research Center. Laboratory and field trials are planned to quantitate the positive production benefits of MCE treatment. A CRADA was established with Goldsboro Milling to test our mucosal competitive exclusion culture with turkeys. The trials should begin in late fall or early winter. A CRADA has been established with EMBREX to further study *in ovo* application of defined and undefined competitive exclusion cultures.

OBJECTIVE C:

Development of improved preenrichment broth for recovery of *Salmonellae* and *Listeriae* from poultry and other food products.

PROGRESS C:

An AOAC precollaborative study was completed demonstrating the efficacy of universal preenrichment broth for recovery of *Salmonellae* from foods. Universal preenrichment was equal to or more effective than the currently used procedure with 17 of 20 foods tested. An AOAC collaborative study is being prepared and will be completed in the winter of 1995.

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PATHOGENESIS, TRANSMISSION, AND CONTROL OF SALMONELLOSIS IN SWINE

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Completion date: June 29, 1997

OBJECTIVE A:

To identify virulence factors important in the pathogenesis of *Salmonella choleraesuis* and *Salmonella typhimurium* in swine.

PROGRESS A:

We attempted to identify antigens which may elicit a specific immune response to *S. choleraesuis*. A cosmid library was prepared to *S. choleraesuis*. The library was amplified as individual clones and we isolated the expressed antigen. Lymphocytes were harvested from 2 animals 16 to 30 days post-challenge with the library *S. choleraesuis* strain for use in blastogenesis assays. Assays have identified 8/610 clones with a minimum 5x stimulation index agreement between both animals. Other clones have also been identified which are specific for the host of origin but do not agree between animals. Protein degradation assays confirm that the antigens are proteins. Currently we are subcloning out of the cosmid vector to identify specific gene segments with the goal to identify the specific protein.

OBJECTIVE B:

To define the epidemiology and transmission of *Salmonella* in swine.

PROGRESS B:

To determine the effect of the route of inoculation, pigs were compared following challenge with 1 x 10^6 CFU *S. typhimurium* by the respiratory route via intranasal instillation (IN) or the oral route via delivery by gelatin capsule (GC) bypassing tonsil and respiratory inoculation. Pigs were necropsied at 2, 4, 6, and 12 weeks post-challenge. At the 2 and 4 week necropsies, more tissue were positive for the IN pigs than for the GC pigs. At 6 weeks post-challenge, all pigs were negative. However, at 12 weeks post-challenge, *Salmonella* were recovered from the ileocolic lymph node of one GC pig. This pig had only one positive rectal swab on day 2 post-challenge. These data suggested that a 10^6 dose may be the minimum dose required to initiate the carrier state. While the data also indicated that IN inoculation afforded wider tissue distribution immediately after exposure when compared to the GC group, no definitive conclusions could be drawn regarding IN vs GC inoculation

involving the long-term carrier except that colonization following GC inoculation will result in positive tissues at least 12 weeks post-challenge.

The effect of route and dose was also conducted with *S. choleraesuis*. Pigs were challenged with 1 x 10^8 CFU by IN or GC inoculation and necropsied at 2, 4, 6, and 12 weeks post-challenge. As observed for *S. typhimurium*, more tissues were positive for the IN vs the GC group through the 6 week necropsy. At 12 weeks the tissue distribution was similar for both groups. Both groups resulted in a carrier status through 12 weeks; however, 3/3 pigs were positive from the IN group vs 2/4 for the GC group. We expected both groups to be positive as a septicemia is typically observed with this dose. Interestingly, fecal shedding was not observed for both groups at week 5 but resumed at week 6 for the GC group and at week 8 for the IN group. Shedding continued for both groups for the remainder of the experiment.

The minimum dose required for initiation of the carrier state with *S. choleraesuis* was determined. Pigs were challenged by IN inoculation with 10^9, 10^6, or 10^3 CFU. By 1 week post-challenge only the 10^6 and 10^9 pigs were shedding. Three pigs per group were necropsied 6 weeks post-challenge. Tissues were positive for both the 10^6 and 10^9 pigs. *Salmonella* was never recovered from either tonsil/nasal/rectal swabs, fecal samples, or tissue at necropsy from the 10^3 group. As previously observed, shedding ceased for the 10^6 group at week 5 but resumed at week 7. The 10^9 pigs ceased shedding at week 7.5 but resumed at week 10. Cessation was again observed at week 10 for the 10^6 pigs. The 10^9 pigs were still shedding 12 weeks post-challenge.

Transmission of *S. choleraesuis* from an infected population to a naive population was studied with *S. choleraesuis*. Previously, we determined that naive pigs become infected with *S. typhimurium* within 2 days post-exposure to the infected group. Twelve pigs were challenged with *S. choleraesuis*. On day 1 post-challenge, 24 *Salmonella*-free pigs were commingled with the infected population. Pigs were necropsied (2 challenged pigs, 4 commingled pigs, and 1 control) 1, 2, 4, 6, 9, and 12 weeks post-challenge. At the 1 and 2 week necropsies, all challenged and commingled pigs were positive for *S. choleraesuis*. The challenged pigs were positive at each necropsy except for week 12 when we were unable to recover *Salmonella* from either of the pigs. At 4 weeks post-challenge, only 3 of the commingled pigs were positive and *Salmonella* was only recovered from 8 total tissues. By 12 weeks post-challenge, only 1 of the commingled pigs was positive for *Salmonella* (2 positive tissues). These data indicate that under well-managed conditions, swine can clear *Salmonella*.

In investigating the role of respiratory inoculation in dissemination of *Salmonella*, we previously determined by use of esophagotomy and intranasal challenge that *S. typhimurium* can reach the gut within 3 h post-inoculation. The tonsil was implicated as a site for invasion. In order to determine what role inoculation into the lung plays in dissemination of *Salmonella*, pigs were inoculated transthoracically with 10^9 CFU *S. typhimurium*. Within 3 h

post-inoculation, all tissues (including tissues collected from the head, thoracic cavity and gut) were positive for *Salmonella*. These data suggested that a wider dissemination of *Salmonella* occurred when the lungs were inoculated and suggested that the respiratory route was an important route of inoculation.

To understand the epidemiology of *Salmonella* in swine, samples collected from 7 swine farms were cultured for *Salmonella*. Samples included fecal samples, rectal swabs, environmental swabs, dust, water, and feed. Two farms were negative for *Salmonella* spp. *Salmonella* spp. were isolated from all other farms and included *S. choleraesuis* var. *kunzendorf*, *S. anatum*, *S. derby*, *S. brandenburg*, *S. typhimurium* var. *copenhagen*, *S. worthington*, *S. agona*, and *Salmonella* untypeable.

OBJECTIVE C:

To define the porcine immune response to acute and chronic *Salmonella* infection focusing on mechanisms to reduce or eliminate the pathogenic organism

PROGRESS C:

The cell-mediated immune (CMI) response was examined to determine the lymphocyte responses following various routes of inoculation with *Salmonella* spp. Preliminary data indicated that the route of infection may affect the development of the CMI response. Pigs infected with *S. choleraesuis* by GC developed an increased antigen specific proliferative T-cell response by day 14 post-challenge, whereas IN inoculated pigs developed an even higher T-cell response at 4 weeks. Both IN and GC routes of inoculation were equally effective at stimulating peripheral blood B-cells. Results of serum ELISA assays suggested that differences between titers of pigs challenged by different routes varied with the antigen. Responses to LPS were greater for the GC pigs than the IN pigs, whereas no difference between groups was observed when soluble antigens were used in the assay. These data indicated that the humoral immune response was modulated by the presentation of different antigens.

A series of experiments was conducted to document the levels of tumor necrosis factor-alpha (TNF) in serum of swine after injection with *Salmonella* spp. endotoxin and after oral or respiratory challenge with live *Salmonella* spp.

Experiment 1: *S. typhimurium* endotoxin (25 ug/kg body weight) was injected intravenously (iv) and serum TNF levels were measured. High levels of TNF (700 IU/ml) at 1-2 h post iv injection correlated with death, whereas lower TNF levels (30 IU/ml) caused a general prolonged state of shock.

Experiment 2: Pigs were injected iv with either *S. typhimurium* or *S. choleraesuis* endotoxin (5 ug/kg). No difference in the ability to induce porcine serum TNF was observed between strains.

Experiment 3: Pigs were inoculated with 1x 10^4 CFU *S. typhimurium* x4232 either orally by gelatin capsule (GC) or by intranasal (IN) inhalation. A late occurring serum TNF

response (17 IU/ml) was measured at 6 weeks post IN inoculation. No serum TNF response was detected in GC inoculated pigs.

Experiment 4: Pigs were inoculated with 1 x 10^6 CFU *S. typhimurium* x4232 as in Exp. 3. Challenge with this medium dose inoculum induced a prolonged peak serum TNF response (37 IU/ml) between 2-and 4-weeks post IN inoculation. Again, no serum TNF activity was detected from GC inoculated pigs.

Experiment 5: Pigs were inoculated IN with 1 x 10^3, 1 x 10^6, or 1 x 10^9 *S. choleraesuis* x3246. A dose-dependent serum TNF response was observed. Therefore, route of inoculation played an important role in determining the porcine TNF response to *S. typhimurium* and *S. choleraesuis* infections. Serum TNF may have been important to the clearance of *S. typhimurium* following respiratory exposure, but was unimportant to clearance of orally presented *S. typhimurium* inoculum. In addition, serum TNF had no apparent effect on clearance of IN or GC inoculated *S. choleraesuis*.

OBJECTIVE D:

To identify methods to control *Salmonella* in swine

PROGRESS D:

We attempted to raise pigs free of *Salmonella* spp. to 6 weeks of age for research purposes by use of isolated weaning techniques. Three methods were investigated over 16 trials. The pigs were not medicated or vaccinated. For method 1, pregnant gilts were transported to isolation facilities and allowed to farrow. In 2 of 4 trials, 130 pigs were weaned at 21 days of age and raised free of *Salmonella* spp. For method 2, gilts were farrowed in the source herd and, at weaning, pigs were transported to isolation facilities at 14 to 17 days of age. In 4 of 5 trials, 68 pigs were raised free of *Salmonella* spp. For method 3, gilts were farrowed in the source herd and, at weaning, pigs were transported to isolation facilities at 10 to 14 days of age. In 7 of 7 trials, 172 pigs were raised free of *Salmonella* spp. These data indicated that the likelihood of infection of pigs with *Salmonella* spp. was markedly decreased by use of isolated weaning techniques and suggested that use of isolated weaning techniques was a practical approach for raising and maintaining pigs free of *Salmonella* spp.

Currently, we are finishing a second isolated weaning trial to determine if pigs can be raised free of *Salmonella* spp. to market weight. Results to date indicate that pigs kept on the source farm became infected with *Salmonella* spp. while pigs transported to NADC and raised in isolation are remaining free of *Salmonella* spp. We are also developing a Hazard Analysis Critical Control Points plan for use on swine farms. A segregated early weaning study involving the reduction/elimination of several swine pathogens is also being conducted.

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CONTROL OF *SALMONELLA* AND *ESCHERICHIA COLI* O157:H7 IN LIVESTOCK DURING THE PREHARVEST PERIOD

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GENERAL OBJECTIVE:

Reduce *Salmonella* and *Escherichia coli* 0157:H7 in cattle, sheep, and swine during the preharvest period using rapid antemortem and possibly postmortem tests by identifying critical control points and effective intervention techniques in the production cycle.

OBJECTIVE A:

Develop rapid tests for *Salmonella* and *E. coli* O157:H7.

PROGRESS A:

Primers were developed and used in polymerase chain reactions (PCR) to amplify 50 different DNA segments of *S. typhimurium*. The gene domains included flagellum, porins, and fimbria segments of the DNA. These gene domains were selected according to the IBI Pustell DNA program. The selected gene domains were amplified using PCR techniques, constructs were made and put into *E. coli* for expression. All 50 gene domains were successfully expressed and the recombinant proteins isolated by gel electrophoresis. These recombinant proteins (r-proteins) were screened using anti-*Salmonella* hyperimmune serum and all were reactive--indicating they had potential for use in developing tests for *Salmonella* detection.

These r-proteins were reacted in Western blot assays against sera from sheep and cattle that had been infected experimentally with *S. typhimurium*. Four of the 16 flagellum r-proteins reacted with sera from all the *Salmonella*-challenged animals. These four r-proteins are being used to develop a serologic test to detect *Salmonella* antibodies in infected animals. Also, r-protein associated with the outer membrane protein C was also reactive with all the sera from the *Salmonella*-challenged animals and this protein is also being used developing a serologic test for *Salmonella* antibodies in infected animals. Polyclonal antibodies against 24 of the flagellar, porin, and fimbria r-proteins have been produced and are being tested for use in an ELISA test for *Salmonella* antigens.

Another set of primers were developed that successfully detected 35 different *Salmonella* serotypes (all that were tested). The primers did not detect 24 non-*Salmonella* gram-negative

bacteria. Thus, these primers show promise for use in detection of *Salmonella* in tissues, fecal material, and many other types of samples.

We are currently searching for herds of cattle that are infected with *Salmonella* so that blood, fecal, and tissue samples from infected cattle can be obtained to screen the r-proteins, primers, probes we developed, and to verify the usefulness of the serologic, antigen capture, and PCR
tests currently under development.

Recently we began extensive efforts to develop monoclonal antibodies against the r-proteins and other antigens for use in detection of *Salmonella* and *E. coli* O157:H7.

We are in the early phases of developing rapid tests for *E. coli* O157:H7. Currently we are developing probes for detection of *E. coli* O157:H7 using subtraction library techniques.

OBJECTIVE B:

Apply the tests we are developing to help us identify critical control points in the production cycle and determine intervention techniques for reducing these specific pathogens.

PROGRESS B:

Ground beef of 2 different leanness values, 80 and 95%, were assayed and found to be free of salmonellae and other culturable bacteria. Each type of beef was then inoculated with 1,000,000 colony forming units (cfu) of *S. typhimurium* per gram of beef. Samples were equally divided into 2 temperature storage groups, 4 and -20°C. All samples maintained at least 50% viability through 7 days of storage. For diagnostic purposes, where prolonged storage is necessary, the 28-day values showed convincingly that freezing samples allowed more viable *S. typhimurium* to be detected than in the samples which were refrigerated.

Transmission of *Salmonella* was studied by experimentally contaminating approximately one-year-old steers with three serotypes of *Salmonella*. The three serotypes used were *Salmonella typhimurium*, *S. newport*, and *S. arizonia*. Each serotype was mixed into fresh bovine feces at 40,000,000 organisms per gram. The fecal material was applied bilaterally to the skin of the front and hind legs to the mid-metatarsal/carpal area and to the skin (1' x 1.5' area) just behind the last rib. The application was repeated one week later and the cattle killed and necropsied one week after the second application. Lateral transmission of *S. typhimurium* and *S. newport* occurred between the feet and legs of all cattle and metastatic infection of *S. typhimurium* occurred in the majority of cattle, as measured by isolation of the organism from the ileal cecal lymph nodes. *Salmonella* could not be isolated from the feces of the cattle irrespective of whether or not *Salmonella* was present in the ileal cecal lymph nodes, colon, or rectum.

We hope to use the tests that are being developed for the following series of epidemiologic studies:

1. Determine the phase(s) of the beef-production cycle (birth to slaughter) at which cattle are infected or contaminated with salmonellae.

2. Determine the critical control points in the cattle-production cycle, common sources, and mechanisms of the infection of cattle during various phases of the beef-production cycle.

3. Develop a monitoring procedure to determine the *Salmonella* contamination and infection status at farms, feedlots, preslaughter, and slaughter that can be used as part of a trace-back system.

4. Develop methods to reduce sources of salmonellae contamination and transmission of infection at critical points in the cattle-production cycle.

5. Develop (at MARC and commercial herds) prototype methods to reduce and control *Salmonella* in a preslaughter control program.

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E. COLI O157:H7 IN CATTLE AND SWINE

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OBJECTIVE A:

Provide knowledge required to develop means to reduce shedding of zoonotic *E. coli* in livestock.

PROGRESS A:

We have characterized the shedding of *E. coli* 0157:H7 in experimentally infected cattle. Fecal shedding persisted longer in young cattle than in adult cattle, but shedding levels varied among animals within both age groups. The infectious dose for adult cattle was high ($\geq 10^7$). In experimentally infected animals, the greatest number of 0157:H7 was in the large bowel and feces. Infection of cattle with 0157:H7 did not prevent reinfection with the same strain.

We previously demonstrated that *E. coli* strains, including 0157:H7, grew better in rumen contents from fasted animals than from rumen contents from well-fed animals. Fasting increased shedding of non-pathogenic *E. coli* 1,000- to 10,000-fold. We will test if fasting also increases shedding of 0157:H7. These results have implications for feeding and management techniques immediately prior to slaughter.

We initiated a collaborative effort with Tuskegee University to determine the prevalence of 0157:H7 in cattle feces immediately before slaughter. This study will provide a base-line of 0157:H7 infection in cattle as they enter the food chain as well as a reference point for determining if certain management practices reduce the level of 0157:H7 shedding in cattle entering the slaughter plant.

OBJECTIVE B:

Develop vaccines that prevent *E. coli* infections in livestock.

PROGRESS B:
Shiga-like toxins (SLT) are involved in hemolytic uremic syndrome, which may follow O157:H7 infections in humans, and edema disease of swine. We used swine as a model to determine if vaccination can prevent edema disease caused by Shiga-like toxin+ *E. coli*

(SLTEC). We have previously shown that subclinical edema disease, which decreases rate of gain, can be prevented by a recombinant DNA produced mutant SLT toxin (antigenic, nontoxic) vaccine. We have now determined that this vaccine also prevents clinical edema disease. In these experiments, unvaccinated pigs had 40% mortality and 100% morbidity. Vaccinated pigs had no mortality and little morbidity. This demonstrates that mutant toxin vaccine can prevent both mild and severe forms of disease caused by SLTEC.

We plan to determine the prevalence and economic impact of edema disease in swine by correlating levels of anti-toxin antibodies with edema disease-associated mortality and decreased rate of gain in commercial swine units. This will demonstrate if serology predicts prevalence and economic impact of SLTEC in swine and other species.

OBJECTIVE C:

Identify virulence mechanisms of *E. coli* that permit colonization in gastrointestinal tract of livestock.

PROGRESS C:

We are trying to determine where 0157:H7 persists in the alimentary tract of cattle. Experiments in progress will determine if 0157:H7 adheres to and colonizes the intestinal mucosa of cattle. Also, we plan to examine the role of rumen microbes in the persistence of 0157:H7. This information is needed to identify which virulence mechanisms play a role in colonization and long-term shedding of 0157:H7 in cattle.

OBJECTIVE D:

Develop rapid detection techniques that identify *E. coli*-infected cattle and swine.

PROGRESS D:

We developed a Multi-plex PCR assay that can identify 4 common toxin genes (LT, STa, STb, SLT) present in *E. coli* pathogenic for swine or cattle. This PCR-based assay rapidly identifies both enterotoxin+ and Shiga-like toxin+ *E. coli*.

We are using single-stranded conformational polymorphisms (SSCP) to detect variability in virulence genes of *E. coli*. SSCP can rapidly detect a single base pair mutation in a PCR amplified gene and is well suited for rapidly examining large numbers of strains. We have determined that SSCP in the eae gene can differentiate enteropathogenic *E. coli* from enterohemorrahagic *E. coli*. We are also using SSCP to determine if variations in the eae gene occur in various 0157:H7 strains.

OBJECTIVE E:

Characterize strains of *E. coli* O157:H7.

PROGRESS E:

We used DNA probes specific for SLT-I and SLT-II to characterize O157:H7 isolates obtained from FSIS. In previous years, we have also profiled isolates from the APHIS NAHMS surveys.

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CONTROL OF *CAMPYLOBACTER JEJUNI* IN POULTRY

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CRIS #: 6612-42000-018
FSIS #: I-83-61
Completion Date: February 13, 1999

OBJECTIVES A:

Develop a vaccine for chickens that will result in a reduced number of chickens carrying *Campylobacter*.

PROGRESS A:

A vaccine has been developed that is a genetic fusion of the flagella from *Campylobacter jejuni* and a portion of the heat labile toxin (LT) of *Escherichia coli*. The toxin makes the vaccine more active when given orally. This vaccine was used to treat chickens that were subsequently given a dose of *C. jejuni*. Almost 50% fewer vaccinated birds had the organism at time of sampling compared to non-vaccinated birds. Tests are continuing to determine if the efficacy can be increased.

OBJECTIVE B:

Determine components of *C. jejuni* that are necessary for its ability to reside (colonize) in chicken intestines that may serve as vaccine candidates.

PROGRESS B:

A protein from the cell surface of *C. jejuni* has been cloned and its DNA has been sequenced. The protein has a high degree of similarity with a binding protein from another organism. This may be important in the colonization methods used by *C. jejuni* or it may just be a means for the organism to acquire nutrients. Experiments are being performed to further characterize this protein.

OBJECTIVE C:

Assess chicken-inherited resistance to colonization by *Campylobacter* and *Salmonella*. By identifying inbred lines with inherent resistance, create commercial broilers carrying fewer *Campylobacter* and *Salmonella*.

PROGRESS C:

We have been testing inbred lines of chicks to assess differences in levels of colonization by both *Campylobacter* and *Salmonella* spp. We have previously reported on the successful identification of resistant leghorn-lines of chickens. We have progressed to testing broiler-type chickens provided by a large commercial breeder. Additional lines of broilers and/or longer term studies may still be required to make further progress.

OBJECTIVE D:

Develop antagonistic flora inhibitory to *Campylobacter* colonization.

PROGRESS D:

We have isolated and identified 12 obligate anaerobic strains and 24 facultative isolates from our mucosal competitive exclusion (MCE) flora. We shall continue to enlarge the numbers of these bacterial antagonists and test both *in-vitro* and *in-vivo* efficacy against *Salmonella* and *Campylobacter* colonization. We have further identified and characterized *in-vivo* antagonism of *Saccharomyces* spp. upon *Salmonella* and *Campylobacter* colonization. This defined antagonist has consistently reduced the colonization of these two pathogens in our chick-isolation unit studies. A patent application for this novel process has been filed. An exclusive license for our MCE patent has been applied for by a large company and is going through the Federal Register review process before being granted to a commercial interest.

OBJECTIVE E:

Develop methodology to simplify isolation and enumeration of *Campylobacter* in poultry and poultry products.

PROGRESS E:

Widespread characterization of the presence of *Campylobacter* throughout the food production and processing industry has been hampered because of methodology for its isolation and confirmation. Previously, enrichment methods required agitation, multiple additions of selective antibiotics, multiple increases of incubation temperature, and repeated introduction of the requisite microaerobic atmosphere. Cooperatively, we have developed and tested a new enrichment method which does not require these steps. Enrichment culture is then plated onto Campy-Cefex and incubated under microaerobic atmosphere. Characteristic colonies are identified by colonial morphology and phase-contract microscopy and, are confirmed by a latex agglutination assay. Using this enrichment/plating procedure we were able to greatly simplify and approximately double the sensitivity for recovery of *Campylobacter* from poultry carcasses.

Further, we have been cooperating in the development and testing of an automated enzyme linked fluorescent antibody (ELFA) assay for the organism. Results gathered indicate that within approximately one hour of analysis time we can discriminate flocks of chickens having heavy vs. low levels of *Campylobacter* colonization. This ELFA procedure also is being studied for applications with poultry products.

OBJECTIVE F:

Develop and apply genotyping systems for epidemiological applications.

PROGRESS F:

We are working cooperatively with institutions in developing methods and software required for "fingerprinting" isolates of *Campylobacter*. At present, a Restriction Fragment Length Polymorphism (RFLP) analysis of the flagellar genome has been developed and is being tested in a multicenter study to assess its efficacy and ability to discriminate isolates. This method is being applied to isolates provided by the FSIS in their baseline studies to determine potential epidemiologic linkage. In a similar thrust, but necessarily different focus, development of a "ribotyping" system is proceeding. We have been working with DuPont corporation and provided them with isolates from poultry sources to compare ribotyping patterns with those of human origins. The RFLP and ribotyping systems could replace the currently dysfunctional serotyping systems for *Campylobacter* typing. The combination of these two systems should allow for clear discrimination between clones of closely related isolates, as is the case with using both heat labile and heat stable antigens in serotyping.

PUBLICATIONS:

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FOODBORNE PATHOGENS: LISTERIA MONOCYTOGENES, CAMPYLOBACTER, ARCOBACTER, AND YERSINIA

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CRIS #: 3630-32000-077
FSIS #: I-83-61
Completion date March 8, 1999

OBJECTIVE A:

Develop rapid and sensitive detection techniques.

PROGRESS A:

Recently, bacteria of the genus *Arcobacter*, which are morphologically similar to campylobacters, have been included on the International Classification of Disease as potential food-borne pathogens. Of the 3 species of *Arcobacter*, *A. butzleri* is the species primarily recovered from human clinical cases. We have designed a probe specific for *Arcobacter*, which does not cross-react with any campylobacter-like organisms. We have also designed and tested a probe which is specific for *A. butzleri*. This probe has been used in prevalence surveys (a) to rapidly identify suspect bacterial colonies, and (b) to obtain DNA fingerprints or ribotypes of field strains.

OBJECTIVE B:

Define the epidemiological roles of livestock and poultry as carriers of potential food-borne pathogens.

PROGRESS B:

Previously, we reported on a prevalence study in which we recovered significantly more *Arcobacter* in aborted porcine fetuses than in normal fetuses. As a result of that study, we surveyed 3 ground pork plants for the presence of *Arcobacter*. Recoveries ranged from 0 to 90% positive ground pork samples. Studies are currently being planned to identify factors underlying plant differences in the rate of contaminated pork products. Because *Arcobacter* is similar to *Campylobacter*, and thus may occupy the same ecological niche, poultry products were also surveyed for *Arcobacter*. Turkey and poultry skins were cultured and found to be positive for *Arcobacter*. A collaborative effort with ARS-Athens, GA, showed that *Arcobacter* spp. are frequently, but not always, isolated from broilers when *Campylobacter* spp. are present.

PUBLICATIONS:

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FOODBORNE PATHOGENS: *LISTERIA MONOCYTOGENES*, *CAMPYLOBACTER*, *ARCOBACTER*, AND *YERSINIA*

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CRIS #: 3630-32000-077
FSIS #: I-88-01
Completion date March 8, 1999

OBJECTIVE A:

Develop rapid and sensitive detection techniques.

PROGRESS A:

Listeriolysin O (LLO) is a virulence protein released only by *Listeria monocytogenes*. No other species of *Listeria* synthesize this factor. We have developed a LLO-based ELISA assay to screen serum of cattle experimentally infected with *L. monocytogenes*. Only serum from cattle experimentally infected with *L. monocytogenes* give a positive response; cattle experimentally infected with *Staphylococcus aureus* do not respond. We have developed a sensitive skin test based on LLO to detect sheep infected with *L. monocytogenes*. Sheep experimentally infected with *L. monocytogenes* give a positive skin test; animals receiving other species of *Listeria* do not respond. We do not know if LLO-based assays can distinguish acute versus past infections. Addressing this question would be required before LLO-based assays are used in a preharvest HACCP procedure.

OBJECTIVE B:

To continue to use restriction enzyme analysis in characterizing *Listeria monocytogenes* strains.

PROGRESS B:

We explored the feasibility of plasmid profiles to distinguish strains of *L. monocytogenes*. Because of our past success in distinguishing isolates based on chromosomal restriction enzyme analysis, we compared plasmid profiles following endonuclease digestion. Following endonuclease digestion, more patterns were generated from chromosomal DNA in comparison with the limited number seen in endonuclease-digested plasmids. This argues for the continued use of restriction enzyme analysis of chromosomal DNA in distinguishing isolates of this food-borne pathogen.

OBJECTIVE C:

Evaluate management practices to reduce or eliminate zoonotic food-borne pathogens.

PROGRESS C:

We are participating in a study to evaluate segregated early weaning as a management technique to reduce zoonotic food-borne pathogens in pigs. Piglets (n=500) were sampled as they entered the nursery (approx. 3 weeks of age) and as they exited approximately 4-5 weeks later. Rectal swabs were cultured for *Listeria monocytogenes*, *Campylobacter*, and *Arcobacter*. Preliminary data indicate that piglets are positive for *Listeria* spp. despite routine antibiotic therapy administered as they entered the nursery and a diet of medicated feed.

PUBLICATIONS:

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DIAGNOSIS AND EPIDEMIOLOGY OF BOVINE TUBERCULOSIS

ARS Contact Persons:
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CRIS #: 3630-32000-068
FSIS #:
Completion Date: Sept. 30, 1997

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OBJECTIVE A:

Develop rapid detection techniques to identify infected cattle and cervids.

PROGRESS A:

Current techniques for preharvest diagnosis of bovine tuberculosis were compared. The tuberculin skin test was found to be more sensitive than a gamma-interferon blood tuberculosis test for identification of cattle infected with Mycobacterium bovis. The effects of stress, such as that encountered during parturition or shipping, on tuberculin skin testing and the gamma interferon assay were determined. Reactivity in both tests was markedly reduced in animals injected with drugs which simulate the effects of stress. This could lead to false negative results with both of these tests in stressed animals.

Elk have been experimentally infected with *M. bovis*. Various diagnostic procedures, including skin testing, blood tuberculosis testing, ELISA, culture, and PCR are being performed at regular intervals in the elk to determine the best test or combination of tests for antemortem detection of tuberculosis in cervids.

PUBLICATIONS:

Whipple, D.L., C.B. Bolin, A.J. Davis, J.L. Jarnagin, D.C. Johnson, R.S. Nabors, J.B. Payeur,
D.A. Saari, A.J. Wilson and M.M. Wolf. Comparison of the sensitivity of the caudal fold skin test and a commercial gamma-IFN assay for diagnosis of bovine tuberculosis. Am. J. Vet. Res. (In Press).

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OSTEOMYELITIS-SYNOVITIS IN TURKEYS

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CRIS #: 6226-32000-002
FSIS #: I-92-2
Completion date June 18, 1996

OBJECTIVE:

To determine the etiology of turkey green-liver/osteomyelitis complex (TOC) and formulate management practices that will lead to the elimination of the problem.

PROGRESS:

Two turkey flocks from farms with above-average condemnation for green-liver/osteomyelitis complex (TOC) were studied throughout a 16-week growout. Green-liver associated TOC was not observed until the turkeys were 9 weeks of age. The incidence of TOC was higher on one farm which also had a higher incidence of air-sacculitis, mortality, and seroconversion to Newcastle disease virus and *Mycoplasma meleagridis*. The turkeys on this farm also had significantly higher average body weights, relative spleen weights, and relative liver weights. Birds on both farms had a high incidence of intestinal lesions and infestation with *Ascaridia dissimilis*. Histological evaluation of green livers revealed hyperplasia of bile ducts, dilation of sinusoids and pigment-containing Kupffer cells, some of which stained positive for iron. Gram-variable, pleomorphic bacteria were isolated from livers, osteomyelitis lesions, abscesses, granules, humerus fluid, and bone tissues only after extended incubation. *Staphylococcus aureus*, *Escherichia coli*, and *Enterococcus facaelis* were also isolated and identified. This study provided evidence of a complex, chronic condition of otherwise healthy birds, which was associated with a relatively high level of TOC.

Further study and observation of the pleomorphic organisms isolated from green livers, synovitis lesions and bones led to speculation that these organisms are the L-forms or cell wall deficient (CWD) variants of some of the opportunistic bacteria isolated from the lesions. This hypothesis has been supported by the isolation of CWD bacteria from the lesions when samples are grown on hypertonic medium. The same samples appear sterile on blood agar or brain heart infusion agar. These CWD variants are sometimes cultured from normal turkey livers and bones. Preliminary attempts to quantitate the CWD variants from a small number of samples resulted in detection of a threefold higher number of organisms in green livers from birds with TOC lesions compared to green livers from birds without TOC lesions. In this study the CWD variants were not detected in normal liver tissues. The majority of these isolates appear to be variants of the same bacterial species associated with TOC lesions in our studies and in the literature. It is generally thought that the tissues of healthy animals are sterile. However, in animals continually challenged by an environment saturated with

potential pathogens, the ability of the immune system to destroy invaders may be overwhelmed. CWD variants may be the result of an inefficient attack on these invaders by phagocytic cells. Their ubiquitous presence in tissues, if confirmed, may represent a means for the long-term persistence of organisms in the animal; the reversion of these forms to cell-walled, infectious parent bacteria might be precipitated by any form of stress. Therefore, the route of exposure and initial portal of entry of these organisms may be different in each case, and may not be important to the outcome. The important factor in a population in which most birds are harboring high numbers of bacterial pathogens in their tissues would be their relative ability to kill or contain those pathogens. Hence, we have become interested in the phagocytic and bactericidal functions of turkey heterophils and macrophages. We are currently studying the immunological responsiveness of lame and normal turkeys from a local farm. It is hoped that this project will define some differences between the immune functions of birds with osteomyelitis and without osteomyelitis.

We have completed a study of the effects of feed and water withdrawal on the incidence of green livers in turkeys over time. It is thought that green livers may sometimes be the result of bile stasis caused by feed withdrawal prior to processing. In our study, no green livers were seen when feed, water, or feed and water were withdrawn at 4-hour intervals over a 32-hour period.

PUBLICATIONS:

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Rath, N.C., G.R. Bayyari, J.N Beasley, W.E. Huff, and J.M. Balog. 1994. Age related changes in the incidence of tibial dyschondroplasia in turkeys. Poultry Science 73:1254-1259.

Bayyari, G.R., W.E. Huff, R.A. Norton, J.K. Skeeles, J.N. Beasley, N.C. Rath, and J.M. Balog. A longitudinal study of green-liver osteomyelitis complex in commercial turkeys. Avian Diseases. (Accepted for publication).

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turkeys. 94th General Meeting of the American Society for Microbiology. Las Vegas, NV. (Abstract).

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TOXOPLASMA GONDII - RECOMBINANT ANTIGEN

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CRIS #: 1265-32000-045
FSIS #: I-94-10
Completion Date: September 25, 1995

OBJECTIVE:

Develop low-cost assay to detect antibodies to *Toxoplasma*.

PROGRESS:

The objective of this project is to develop a low-cost assay to detect antibodies to *Toxoplasma gondii* in naturally infected animals. In order to have reliable antigen available in large quantities, two recombinant antigens of *T. gondii* (H4/GST and H11/GST) were evaluated to detect *T. gondii* antibodies in pigs. Both of these antigens were initially characterized by Drs. Alan Johnson (Sydney, Australia) and A. Tenter (Hannover, Germany). Antigens H4 and H11 produced by Drs. Johnson, Tenter, etc. were moderately to somewhat reactive with early infection sera. The results were highly variable from animal to animal. These antigens were deemed "not appropriate" for serodiagnosis of *Toxoplasma* infected swine.

Four antigens from *T. gondii* bradyzoites produced by Drs. S. Parmely, and Jack Remington of Stanford University, California, have been evaluated in the same manner. Two of these four antigens were highly reactive with early infection sera. In combination, these two antigens produced strong reactions with all of the sera tested up to a year after infection, although there was some decline in reactivity associated with duration of infection. Since one weakness of ELISA with whole antigen is a significant lag period in detecting early infections, the recombinant antigens show promise for serodiagnosis and are worthy of further investigation. Additional work needs to be done to determine sensitivity and specificity.

PUBLICATIONS:

None

PORCINE TOXOPLASMOSIS - NATIONAL PREVALENCE

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CRIS#: 1265-32000-045
FSIS #: I-89-94
Completion Date September 30, 1995

OBJECTIVE:

Determine the prevalence of toxoplasmosis in swine and develop control procedures.

PROGRESS:

In a serologic survey, 23.9% of 11,842 commercial pigs slaughtered in 1983-84 throughout the United States had antibodies to *Toxoplasma gondii* by the use of a modified agglutination test (MAT). A study was done to determine if pigs seropositive to *T. gondii* actually harbor viable *T. gondii* in body tissues. For this, heart tissue from 1000 Iowa sows were bioassayed for the presence of *Toxoplasma* and compared with serology results from the same pig. Twenty-two percent of sows killed in 1989-1992 had *T. gondii* antibodies and viable *T. gondii* was isolated from the hearts of 170 of these pigs.

The sensitivity and specificity of MAT and various other serologic tests (latex agglutination test (LAT), indirect hemagglutination test (IHA), dye test (DT), and ELISA) for antibodies to *T. gondii* were compared in these 1,000 sows using the isolation of viable *T. gondii* as the definitive test. The percentage of samples diagnosed as positive for each of the serologic tests was: MAT = 22.2% (titer $\geq$ 1:20), IHA = 6.4% (titer $\geq$ 1:64,), LAT = 10.4% (titer $\geq$ 1:64,), and ELISA = 24.1% (OD > 0.360). The sensitivity and specificity of these tests were calculated respectively to be : 82.9% and 90.29% for MAT, 29.4% and 98.3% for IHA, 45.9% and 96.9% for LAT and 72.9% and 85.9% for ELISA. The dye test was run at 1:20 dilution on only 893 sera because of bacterial contamination and the presence of anticomplement substances. Dye test antibodies were found in 17.8% of the sera and the sensitivity and specificity of the DT were 54.4% and 90.8%, respectively. Thus, the MAT had the highest sensitivity among all serologic tests used.

PUBLICATIONS:

Dubey, J.P., Thulliez, P., and Powell, E.C. 1955. *Toxoplasma gondii* in Iowa sows: Comparison of antibody titers to isolation of *T. gondii* by bioassays in mice and cats. J. Parasit. (In press).

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RECOMBINANT ANTIGEN FOR DIAGNOSIS OF BOVINE CYSTICERCOSIS

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CRIS #: 1265-32000-030
FSIS #: I-90-5
Completion date June 30, 1994

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OBJECTIVE:

To develop a recombinant antigen for the serodiagnosis of bovine cysticercosis

PROGRESS:

We previously identified and purified an antigenic fraction from the cyst fluid of *Taenia hydatigena* (designated THFAS) that demonstrates applicability as a sensitive and specific reagent for the diagnosis of bovine cysticercosis. Rabbit antiserum to THFAS identified a homologous antigenic protein in the cyst fluid of the cestode *Taenia crassiceps*. The distribution and localization of this highly conserved 10 kDa THFAS antigen was therefore investigated using the metacestode stage of *Taenia crassiceps*. The 10 kDa protein was secreted by the metacestode parasites into media during 1 to 7 days of *in vitro* cultivation. The protein was present in high concentrations in the cyst fluid and could be extracted also from the cyst wall tissue. Immunohistochemical studies localized the 10 kDa protein to intramuscular organelles located below the tegument of the cyst wall and was not detected on the outer surface of the parasite by either immunohistochemistry or immunofluorescence. It is not clear if the protein is synthesized in the cyst wall organelles and then secreted both internally and externally or synthesized elsewhere and transported through the cyst wall within these organelles for secretion into the external environment.

A cDNA expression library was constructed using poly A mRNA purified from *T. crassiceps* cysticerci and screened with rabbit antisera to THFAS or with bovine antisera to *Taenia saginata* to identify a recombinant THFAS homologous epitope. Positive clones were identified which reacted with both antisera. One strongly reactive clone designated lambda TCA-2, generated a fusion protein 130 kDa in size and contained a cDNA insert 0.45 kb in length. The antigen from lambda TCA-2 interacts strongly with sera from cattle experimentally infected with *T. saginata*, and does not cross-react with sera from cattle infected with *Fasciola hepatica* or with other common gastrointestinal cattle parasites. The 0.45 kb insert was subcloned into a second vector and expressed in order to facilitate purification of the recombinant antigen as a fusion protein containing the maltose binding protein. Preliminary results using both Western blot analysis and ELISA demonstrate that *T. saginata* antibodies from naturally-infected cattle can also be detected with this cloned antigen.

Subsequent to ELISA testing, the cDNA expression library was rescreened with TCA-2 and a complete cDNA sequence, designated pTCA5.5, was identified. This clone has been sequenced and both Southern and Northern blot analyses performed. The putative 0.45 kb TCA-2 sequence was determined to be 350 base pairs in length and representative of the partial cDNA sequence when compared by Northern blot analysis Identification and sequencing of the full length sequence has allowed us to identify similar sequences from other Taenids. To this end, we have been able to obtain *T. saginata* gravid proglottids from which we have isolated poly A mRNA and constructed cDNA libraries. These libraries were immunologically screened and several positively hybridizing clones were amplified and sequenced. From the sequence data, several regions which were homologous between *T. saginata*, *T. hydatigena* and *T. crassiceps* antigens were identified. Peptides have been chemically synthesized from regions designated as putative antigenic epitopes. These peptides have been tested against sera from experimentally-infected animals for their potential to bind antibody. Results indicate that multiple sites are necessary for proper antibody binding. Further differential screening of the *T. crassiceps* cDNA library has identified several additional clones containing partial cDNA sequences which appear distinct from TCA-2 but which generate an immunologically cross-reactive recombinant antigen. Work is presently underway testing one of the new recombinant peptides designated TCA-6.2, for diagnostic capabilities.

Initial trials in our laboratory have demonstrated that reactivity between sera immunoglobulins from cysticercosis-uninfected naturally raised cattle and bacterial contaminants in the recombinant antigen sample TCA2-MBP resulted in elevated ELISA values. Pre-absorbing the sera with bacterial cell lysate prior to incubation with TCA2-MBP reduced this reactivity with negligible effect on specific reactivity with sera immunoglobulins from cysticercosis-infected cattle. More stringent procedures for the purification of the recombinant antigen are underway. Purified recombinant antigen from the intact fusion protein was provided to FSIS scientists for testing.

FSIS, under the direction of C. Andrews, evaluated the performance of TCA2-MBP in a serodiagnostic ELISA using sera from 3 groups of cattle: experimentally infected calves, normal slaughter cattle, and cysticercosis suspect slaughter cattle. The recombinant antigen was comparable to native antigen fraction ThFAS in its ability of detect specific antibody response over the first 10 weeks of infection in the experimental group. However, in slaughter-age cattle, the recombinant antigen did not differentiate between the normal and cysticercosis suspect groups. The serologic response to the recombinant antigen by both these groups was twice that of the suspect group to the native antigen fraction. The results of the evaluation suggest that the antigen contains multiple antigenic epitopes, one of which is targeted during the early stages of the infection, plus one or more epitopes which are recognized by "normal" adult cattle.

Additional studies were initiated based on the assumption that contaminating proteins from the *E. coli* host were responsible for the non-specific response in adult cattle. A commercial *E. coli* protein extract was provided by ARS for the purpose of determining whether it could

block non-specific antibody responses. A subset of sera from each cattle group was used to investigate the effect of preincubating the sera with the extract. Titration of the extract determined that a concentration of 1% protein lysate dramatically reduced background serologic responses in slaughter sera. Concentrations above this had only slightly greater effects.

The usefulness of the diagnostic fusion protein from *T. crassiceps* larval cysts was evaluated as a potential protective antigen for *Taenia* infections in a small animal model. Mice were immunized with a variety of *T. crassiceps* larval cyst antigens, including the diagnostic fusion protein, then challenged with 5 non-budding *T. crassiceps* cysts intraperitoneally and bled biweekly to track antigen specific antibody titers and peripheral blood eosinophil counts. Eosinophil levels increased 5 fold over control levels in that group immunized with the diagnostic fusion protein. Mice were sacrificed 8 weeks after the challenge infection and the number of intraperitoneal cysts counted to assess the level of protection afforded by immunization with the diagnostic fusion protein as compared to immunization with other larval antigens and controls. Results indicated greater than 95% protection of mice immunized IP with the recombinant antigen relative to mice immunized by other methods. Flow cytometric analysis of peripheral white blood cells and cells which have infiltrated the peritoneal cavity in response to immunization and challenge demonstrate that a shift from predominantly CD4 to predominantly CD8 lymphocytes occurs in the peritoneal cavity of immunized and infected mice. Cell populations which surround and kill larval cysts have been analyzed by immunohistochemical techniques and light and electron microscopy and confirm these findings. These studies in the small animal model suggest cell activation and proliferation patterns which may be useful in monitoring the development of immunity in immunization trials in a large animal system.

PUBLICATIONS:

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EPIDEMIOLOGY AND CONTROL OF TRICHINAE IN THE NORTHEAST (NEW JERSEY/NEW ENGLAND/OHIO)

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CRIS #: 1265-32000-045

APHIS Contact Person:
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OBJECTIVES:

1. To validate the use of the ELISA test as a tool for identifying trichinae-infested premises.

2. To associate risk factors with the presence of trichinae in pigs (for possible HACCP programs).

3. To study control procedures, management methods and other factors which can be used to eliminate trichinosis and maintain trichinae-free status.

This project has been initiated as a cooperative effort between ARS and APHIS and has received the endorsement of the National Pork Producer's Council and local affiliates. Protocols have been designed in cooperation with state veterinarians in the locations chosen for the study. The study will be done in several parts, first to associate risk factors with trichinae infection, then to identify foci of infection, and eventually to provide the producer with answers as to how to become and/or remain trichinae-free. In the first part (objective #1), serum samples will be obtained by state veterinary personnel and will be tested for trichinae infection. In addition to serum collection, questionnaires on management factors will be completed at on-farm collection sites. In some cases, when positive serum samples are obtained, infections will be verified on the farm and supplemental epidemiologic evaluations performed to identify mode(s) of transmission. Based on the results of these studies, statistical evaluations will be performed to assign and weight risk factors for trichinae infection. Understanding these risk factors and their relative importance will be valuable information in knowing where infections might exist and under what circumstances farms could be considered risk-free (objective #2). To assist farmers in becoming or remaining trichinae-free (objective #3), we will enroll farms in a volunteer study to examine the relative role of rodents, wildlife and other sources of infestation with trichinae. We will identify the modes of transmission on a minimum of 20 premises, then institute management changes to assess the potential for establishing trichinae-free status. In-depth studies will be conducted on these premises, including rat and other small mammal trapping and an evaluation of other management procedures. All premises will be tested by serology over

time to assess the status of trichinosis in the herds. At the conclusion of the study, final recommendations will be made to farmers concerning the best approach to maintaining trichinae-free status.

PUBLICATIONS:

None

CHECK SAMPLE PROGRAM FOR TRICHINAE INSPECTION OF HORSEMEAT

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CRIS #: 1265-32000-039

AMS Contact Person:
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OBJECTIVE A:

To provide training and quality control to a program for the inspection of horsemeat for trichinae.

PROGRESS A:

The ARS administers a training program for the certification of trichinae analysts in horse slaughter facilities. This program is accepted by the European Union and allows U.S. horsemeat to be certified trichinae-free for export to Europe. Two training sessions are held each year for personnel to become certified as trichinae analysts. A total of 46 analysts have been certified to date. On a quarterly basis check samples are prepared and distributed to all certified trichinae analysts for testing. Accurate analysis of these check samples allows for continued certification of these inspectors.

OBJECTIVE B:

To provide data supporting the efficacy of current and proposed methods for the inspection of horses for trichinae.

PROGRESS B:

As a result of a recent outbreak of trichinellosis in France, questions were raised as to the adequacy of current digestion testing of horsemeat for trichinae. ARS is currently conducting experiments, in collaboration with Agriculture Canada and EU scientists, to determine the most effective method for detecting trichinae in horses and in swine. These studies will examine the pooled sample digestion method, using 1, 5, and 10 gram samples and will compare these results to serology testing. Scientists from Agriculture Canada will use the Trichomatic 35. Final test results will be compared with parallel studies being conducted in Germany. From these studies recommendations will be made for the most effective method for testing. Further, these studies will support the validity of current testing methods used in the United States.

FIELD TESTING OF THE ELISA FOR THE SERODIAGNOSIS OF SWINE TRICHINELLOSIS

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OBJECTIVE:

To validate the efficacy of the ELISA test for swine trichinellosis in large scale field trials.

PROGRESS:

This is a cooperative project between ARS, LMD Laboratories, Inc. (a private company), and the government of Romania. A high level of trichinellosis is found in Romania and this is an ideal location for large scale field testing of the ELISA. ARS scientists will work with the cooperators to provide a highly sensitive version of the ELISA test for use in Romania. High background values or false positives in Romanian pigs will be eliminated by altering serum concentrations or increasing the stringency of washing conditions. Once optimized, Romanian scientists will perform large scale testing and confirmation of infection by direct methods. ARS will provide antigen for use in the test and LMD Laboratories will provide the ELISA test kits. In addition, ARS scientists will provide on-site consultation to Romanian scientists during the conduct of the field testing. Results of ELISA testing will be provided to ARS by Romanian cooperators. The results of this study will be a large data set demonstrating the efficacy of serology testing for trichinellosis in swine which can be used in determining the applicability of serology testing in the U.S. and Europe.

PUBLICATIONS:

None

DETERMINATION OF THE SAFETY OF CHLORINE OR OTHER DISINFECTANTS USED IN MEAT AND POULTRY PROCESSING

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CRIS #: 5325-42000-016
FSIS # : I-82-34
Completion Date September 30, 1995

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OBJECTIVE A:

Investigate systematically the casual relationship between chlorination and mutagen formation in food processing water and determine the specific mutagens formed upon chlorination.

PROGRESS A:

Poultry chiller water (PCW) is routinely chlorinated by the U.S. poultry processors to control microbial populations and is considered by many processors to be essential to the safety and quality of ready-to-cook poultry products. Unfortunately, chlorination of PCW forms trihalomethanes and mutagenic compounds that have yet to be completely characterized. A number of chlorinated mutagenic compounds, other than trihalomethanes, have been isolated from simulated PCW. Although the risk of these deleterious compounds hasn't been established, it is desirable to look to new methods of disinfecting PCW and/or reducing the formation of chlorinated products during the chlorination process. Presently, chlorination is the sole process permitted by regulatory agencies. The simulated water was prepared by homogenizing whole chicken in water, 0.2%, to produce a slurry. The isolated mutagens from the chlorination of the slurry were structurally characterized, synthesized and extensively tested with the Ames mutagenic assay procedure. The compounds are extremely potent (about 1600 rev/nmol), direct acting mutagens, and contribute significantly (>85%) to the overall mutagenicity of the chlorinated PCW. These chlorinated compounds represent a new class of compounds that heretofore have not been known to the scientific community. Experiments are under way to devise methods to deactivate these mutagens in the PCW.

OBJECTIVE B:

Investigate potential disinfectants, other than chlorine, of food processing water.

PROGRESS B:

Chlorine dioxide is an alternative sanitizing agent that holds potential for use in a number of food processing plants including poultry processing. Chlorine dioxide is 4 to 5 times more

effective than chlorine in killing the microorganisms normally found in PCW. An investigation on the disinfection efficacy and chemical reactivity of chlorine dioxide has been completed with the cooperation of a chlorine dioxide manufacturing company and the support of the Food Safety Inspection Service (FSIS). Chlorine dioxide produces fewer reaction products than chlorine as well as little if any mutagenic activity as demonstrated by the Ames assay for mutagenicity. Specifically, no trihalomethanes were found in PCW treated with 100 ppm chlorine dioxide. Data collected by ARS scientists and a local chemical company, were used to petition the FDA for limited use of chlorine dioxide in poultry processing plants. Additional data were requested by the FDA which has now been collected and supplied. Approval to use chlorine dioxide as an alternative disinfectant is expected soon.

OBJECTIVE C:

Filtration studies of processing water and chiller brine.

PROGRESS C:

Methods for reconditioning processed meat chiller brines for reuse were further investigated. Prior work showed that microfiltration of the brine using ceramic microfilters with 0.2 to 0.45 micron pores was efficacious for removing bacteria and particulates from the brine. However, the microfiltration flux was considered marginal to unacceptable for commercial application. Pretreating the brine by filtering it through a precoated diatomaceous earth (DE) filter substantially increased the flux in subsequent microfiltrations. DE filtration is relatively inexpensive, compared to microfiltration, hence the added cost of prefiltration may be more than offset by the gains in microfiltration flux. Tests were conducted in 2 plant locations which process different kinds of products. The DE filtration was accomplished using a plate-and- frame filter. The filter elements were made of paper which was impregnated with DE, providing a nominal pore opening of 0.3 microns. Tests were run using no DE precoat or DE body feed, with precoat only, with body feed only and with combined precoat and body feed. The plate-and-frame configuration is a through-flow configuration, and without DE precoat or body feed the element plugged very rapidly, because the impregnated filter had very little capacity to absorb particulates. As expected, the DE precoat increased the capacity of the system to contain filterable solids prior to plugging. Over the range tested the precoat and body feed showed similar results for increasing filtration capacity based upon the ratio of DE to brine. Combining precoat and body feed further increased the filtration capacity of the system. When body feed was used, the filtration capacity was only limited by the build-up of sludge in the plate-and-frame filter. Prefiltration appears to increase the subsequent microfiltration flux up to 3-fold. This is not unexpected because the DE-impregnated filter element has a 0.3 micron average pore size, which is in the microfiltration range. Studies are in progress to verify the microbial filtering ability of the DE impregnated plate-and-frame filter system. Confirmation of the filtering capacity of the DE filter, with in- plant studies, will be tested.

PUBLICATIONS:

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Ng, K.C., Huxsoll, C.C, and Tsai, L.-S. Treatment of Poultry Chiller Water by Flocculation. J. Food Process Eng. (Accepted for publication).

CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON POULTRY CARCASSES WITHIN THE PROCESSING PLANT

ARS Contact Persons:
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CRIS #: 6612-41420-001
FSIS #: I-82-27
Completion Date: September 30, 1995

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OBJECTIVE A:

Determine the effects of multiple stage scalders on bacterial counts of carcasses.

PROGRESS A:

Scald water and whole carcass rinse samples were collected on 9 occasions in a commercial broiler processing plant running a conventional single-tank 2-pass scalder and a 3-tank 2-pass counterflow scalder on adjacent processing lines. Counts of aerobic bacteria and Enterobacteriaceae were significantly reduced in water in the third tank of the counterflow scalder compared to the conventional scalder. Counts of aerobic organisms were significantly lower on carcasses from the 3-tank scalder, but Enterobacteriaceae counts were not different. Inorganic, organic, and total solids were reduced in the third tank of the counterflow scalder compared to the conventional scalder. There was no significant difference in the microbiological quality of carcasses due to scalding treatment when sampling was done after the last picker.

OBJECTIVE B:

Develop computer simulation of carcass scalding for analyzing the operation of a scalder.

PROGRESS B:

Computer programs were developed to study the fate of bacteria suspended in water during scalding. One- and 3-tank scalder configurations were combined with 3 models of water mixing: perfect mixing (bacterial concentration the same at all points within a tank), 1-pass cell mixing (1-pass tank divided into cells with cell size equal to water volume per carcass, with perfect mixing between adjacent cells), and 2-pass cell mixing (same as 1-pass cell mixing, but with 2 lines of carcasses in each tank with mixing between adjacent cells in the 2 lines. Common commercial scalding conditions were used in calculations. The models can be used to calculate conditions for maximum bacterial reduction in typical industrial scald tanks.

OBJECTIVE C:

Evaluation of carcass cleaning machine and spray scalder.

PROGRESS C:

A CRADA has been developed and approved with an equipment manufacturer to build a spray scalder. The equipment has been completed and after minor modifications, installed in our pilot facility. Preliminary test have been initiated to determine the range of temperature control available.

A CRADA has been developed and approved with an equipment manufacturer to further develop the carcass cleaning machine. A prototype machine has been completed and is awaiting installation in a processing plant.

A CRADA has been developed with a poultry processor for on-line testing and further development of the carcass cleaning machine.

OBJECTIVE D:

Develop microbiological data of picker fingers from rougher to finisher machines.

PROGRESS D:

Rubber picker fingers were profiled for microorganisms in an experiment that was replicated six times. Sample picker fingers were severed from the mounting plates of the first (rougher) and last (finisher) defeathering machines. Microbiological sampling of the fingers did not reveal a significant difference ($P<.05$) in the microorganisms regardless of scalder type. The $\log_{10}$ counts for aerobic, *Enterobacteriaceae*, and *Staphylococcus* were significantly higher ($P<.05$) on fingers taken from pickers operating on the triple-stage scalder line.

OBJECTIVE E:

Pre-evisceration treatments to reduce pathogenic bacteria on poultry.

PROGRESS E:

A spray washer was used to apply acetic acid (2% v/v) solutions to commercially processed uneviscerated carcasses. Vents of the New York dressed carcasses were plugged as they were taken off the processing line to prevent contents of the colon from exuding onto the skin into the chiller during handling and treatment processes. Sample carcasses were hung on the pilot plant shackle line and conveyed through the washer at a line speed equivalent to that of a commercial 6 foot washer installed on a 9600 birds per hour processing line. Temperature of the acid solutions and tap water applied at 827 kPa (120 psi) to the carcasses was

approximately 15°C ± 1°C (≈60°F) for the trial runs. Carcass treatments were: (1) non-washed controls (CONT); (2) tap water spray (SW) with a 25 min drip time followed by a 45 min chill; (3) acetic acid (AA) spray with a 25 min drip time followed by a 45 min chill. Separate carcass chillers, charged with slush ice and water mixtures, were used to chill the treatment carcasses. Bacterial counts were determined by microbiological sampling of rinse water residue derived from the whole carcass rinse procedure. Rinse water samples from the acid sprayed carcasses were neutralized in 0.1 N phosphate buffered saline (pH 7.6) solution. After chilling, *Enterobacteriaceae* counts were reduced by 1.06 $\log_{10}$ on SW carcasses, and by 1.24 $\log_{10}$ on AA treated carcasses and aerobic counts were reduced by 0.98 $\log_{10}$ on SW carcasses, and 0.88 $\log_{10}$ on AA treated carcasses respectively. Microbial counts on CONT carcasses were reduced about 0.5 $\log_{10}$ with a 45 min ice water chill.

OBJECTIVE F:

Post evisceration treatment of carcasses.

PROGRESS F:

Extended chilling times with and without acetic acid and mechanical agitation were studied to determine the effects of treatments on the microbiological quality and incidence of salmonellae on processed poultry carcasses. Extended chilling times with a 0.6% acetic acid chiller medium reduced total aerobes and *Enterobacteriaceae* by 0.2 $\log_{10}$ CFU/ml. The 0.6% acetic acid solution in a static chiller lowered counts by 0.6 $\log_{10}$ more, and when mechanical agitation was incorporated in the trials counts were lowered almost 2 $\log_{10}$. *Salmonellae* incidence of carcasses inoculated with approximately 200 cells of a strain of *Salmonella typhimurium* ranged from a high of 80% for the control and acid groups without mechanical agitation to 40% for the air and acetic acid group to only 6.7% for the mechanically agitated group with acetic acid. Time was only a factor when agitation was not used. The effect of combining sprays, prechill, and chilling with acetic acid on total aerobes was determined. The combination reduced total aerobes by approximately 2 $\log s_{10}$ when compared to the control group. Substitution of chlorine for the acetic acid produced results that were equivalent to the acid treated carcasses.

PUBLICATIONS:

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Dickens, J. A. and A. D. Whittemore. The effects of extended chilling times with acetic acid on the temperature and microbiological quality of processed poultry carcasses. Poultry Sci. (Submitted for publication).

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Shackelford, A. D., A. D. Whittemore, and R. L. Wilson. 1994. Microbiological quality of uneviscerated carcasses after an acetic acid spray rinse and chiller treatment. Poultry Sci. 73 (Supplement 1):24.

Whittemore, A.D. and C.E. Lyon. 1994. Microbiological profile of rubber defeathering fingers and carcasses from processing lines with single and triple stage scalders. Poultry Sci. 73 (Supplement 1):24.

Whittemore, A.D. and C.W. Beard. 1994. Microbiological sampling of egg contents. Poultry Sci. 73 (Supplement 1):105.

POST-CHILL TRIMMING OF POULTRY CARCASSES

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CRIS #: 6612-41420-001
FSIS #: I-94-8
Completion Date: September 30, 1995

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OBJECTIVE A:

Color changes of bruised tissue due to immersion chilling.

PROGRESS A:

The effects of immersion chilling on color changes of bruised tissue were determined by recording objective color values of bruised and unbruised tissue from broiler carcasses. Carcasses were removed from the line in a commercial plant after exiting the last picker. Objective color values (L, a, b) were noted for bruised and unbruised breast and leg tissue before and after chilling. Prechilling and chilling were done in the pilot plant using water and water/ice in tanks with rotating compartments to simulate the tumbling associated with commercial chilling. Redness, a values, of the bruised breast and leg tissue was not significantly altered due to immersion chilling.

PUBLICATIONS:

Lyon, C.E. and J.A. Cason. 1994. Effects of bruising and water chilling on objective color values of broiler tissue. Poultry Sci. 73: (supl. 1): 258. (Abstract.).

CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON RED MEAT

ARS Contact Persons:
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CRIS #: 5438-32000-010
FSIS #: I-94-7
Completion Date May 1, 1996

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OBJECTIVE A:

Develop and evaluate means to rapidly detect animal carcasses accidentally contaminated with feces and to determine the efficacy of reconditioning procedures.

PROGRESS A:

A microbial ATP bioluminescence test (mATP) was developed that will detect relatively high levels of generic bacteria (indicative of fecal contamination). This test is based on the underlying concept that the amount of bacterial ATP (adenosine triphosphate) in a sample is related to the bacterial population. The microbial ATP is detected by a newly devised modification of the firefly luciferin/luciferase reaction. This test is able to distinguish non-microbial from microbial ATP by differential filtration/extraction. The test was validated in the laboratory and in plant settings for use with beef, pork and poultry carcasses. The mATP test is rapid (less than 5 minutes to complete), generic (not specific for just one type of bacteria), field amenable and able to distinguish approximately 1,000 - 10,000 bacteria per cm^2. In the case of poultry, sampling was done with a sponging technique but is expressed on a per ml basis. Correlations between the aerobic microbial plate count method (APC; 48 hours to perform) and the rapid test (5 minutes to perform) were as follows; beef: $r = 0.92$ ($n = 400$), pork: $r = 0.92$ ($n = 320$), and poultry: $r = 0.82$ ($n = 160$).

PUBLICATIONS:

Siragusa, G.R., C.N. Cutter, M. Koohmaraie. 1994. Laboratory and in-plant feasibility studies on the use of microbial ATP bioluminescence assay to detect microbial contamination of beef carcasses. Summary report to the FSIS, 3-8-94.

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Dorsa, W.J. , C.N. Cutter, G.R. Siragusa, and M. Koohmaraie. 1994. Comparison of mATP and aerobic plate count to estimate generic bacterial contamination on beef carcasses. Summary report to the FSIS, 8-5-94.

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CONTROL OF PATHOGENIC AND SPOILAGE BACTERIA ON RED MEAT

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CRIS #: 5438-32000-010
FSIS #: I-12
Completion Date May 1, 1996

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OBJECTIVE A:

Reduce or eliminate pathogenic bacteria on red meats and meat animal carcasses.

PROGRESS A:

Organic acid spray treatment achieved minimal reduction of three strains of viable *E. coli* O157:H7 attached to bovine carcass surface tissue (1.1 to 1.8 $\log_{10}$ colony forming units). Spray treatments were conducted using a pilot scale carcass washing system. The reduction was independent of acid type (whether acetic, citric or lactic acid) but was highly dependent on acid concentration. Maximal reductions were observed with 5% v/v of the respective acid. In the case of the spoilage bacterium *Pseudomonas fluorescens*, $\log_{10}$ reductions were higher than for any of the three strains of *E. coli* O157:H7, ranging from 1.7 to 3.4 $\log_{10}$ units. The effectiveness of acid treatments on the tested strains of *E. coli* O157:H7 was minimal and, in some instances, not greater than the effect seen with plain tap water sprays. However, acid treatments were very effective in reducing the test spoilage organism (>99.9% reduction) and, as in the former case, was independent of acid type. The resulting surface pH of the acid treated tissue indicated that the bactericidal effect due to low pH was a very short lived effect. After 24 h refrigerated storage the pH had returned to the pre-acid treatment levels of the inoculated tissue.

Bacteriocin (nisin) spray treatment was effective in reducing the levels of test bacteria attached to beef surface tissue. Tissues inoculated with up to $\log_{10}$ 4 (10,000) bacteria per cm^2 were spray treated using a pilot scale carcass washing system with either purified nisin or tap water. The bacteria tested were: *Brochothrix thermosphacta* (spoilage bacterium of vacuum packaged meats), Listeria innocua (closely related to the pathogen *L. monocytogenes*), or *Carnobacterium divergens* (a meat spoilage bacterium). Nisin spray treatments reduced bacterial levels 1.8 to 3.54 $\log_{10}$ colony forming units within the first day of treatment and 1.97 to 3.6 log 10 colony forming units after 24 h refrigerated storage. The sensitivity of the test bacteria to nisin was (in order of most sensitive to least sensitive): *B. thermosphacta*, *C. divergens* and *L. innocua*. Although at the present time nisin use as a spray treatment is cost prohibitive, this study clearly demonstrates that a bacteriocin can be applied to carcass tissue

and still retain antimicrobial activity. Future work will address using other and new bacteriocins as well as optimizing production of these agents.

PUBLICATIONS:

Cutter, C.N. and G. R. Siragusa. 1994. Efficacy of organic acids against *Escherichia coli* O157:H7 attached to beef carcass tissue using a pilot scale model carcass washer. J. Food Prot. 57(2):97-103.

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PREVENT THE ATTACHMENT OF FOODBORNE PATHOGENS TO POULTRY SKIN AND MUCUS

ARS Contact Persons:
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CRIS #: 6612-42000-011
FSIS #: I-82-27
Completion Date: September 30, 1995

OBJECTIVE A:

To determine the mechanism of attachment of *Salmonella* to poultry skin.

PROGRESS A:

1. Lipopolysaccharides (LPS) are the main component of the outer cell wall of gram negative bacteria, including *Salmonella*. LPS is involved in bacterial adhesion to substrates. A comparison was made between the attachment to poultry skin of LPS-positive and mutant LPS-negative strains of *Salmonella*. Essentially similar patterns of attachment were observed with both types of strains suggesting that this component of the outer cell wall does not play a significant part in the attachment of salmonellae to poultry skin.

2. LPS was blocked by coating the cell surface with O-specific antiserum to determine if blocking LPS significantly affects the attachment of *Salmonella* to poultry skin. The blocking of LPS seemed to have no significant effect on the binding of *Salmonella* to poultry skin.

3. Viable *Salmonella* cells were killed by irradiation or by treating with 5% formaldehyde to determine if viability is essential for the attachment of salmonellae to poultry skin. There was no significant difference in the rate of attachment of live cells and dead cells (either irradiated or treated with formaldehyde) indicating that viability is not required for attachment to poultry skin.

4. The cell surface was altered by growing *Salmonella typhimurium* in a chemically defined medium. The rate of attachment of these cells was compared to that of cells grown conventionally in brain heart infusion broth. There was no significant difference in the rate of attachment of these two cultures to poultry skin.

Work is in progress to identify the specific location of salmonellae attached to poultry skin by use of confocal laser microscopy. It is anticipated that such a determination may shed some light on the mechanism of attachment. It might also provide information useful to evaluating the potential efficacy of bacteriocides.

OBJECTIVE B:

To determine if *Campylobacter* which are free-swimming in the mucin layer of the intestine may attach to skin differently than *Salmonella* which are not free-swimming.

PROGRESS B:

It was determined that *Campylobacter* on poultry skin react to rinsing in the same manner as *Salmonella*, suggesting that the mode of attachment is similar. Recovery upon repeated rinsing is sporadic. We could not conclude from the study whether all *Campylobacter* detach upon rinsing, and some reattach and are not enumerated the first time, or whether rinsing gradually loosens the association of bacterial cell with skin and causes sporadic releases.

OBJECTIVE C:

To assess the quantitative and qualitative presence of *Arcobacter* in poultry carcasses.

PROGRESS C:

Arcobacter has been shown to be a causative agent in human disease. It has been isolated from the environment and various animals. *Arcobacter* spp. is related to the genus *Campylobacter* and differs primarily in its ability to grow aerobically. This study showed that *Arcobacter* spp. are frequently, but not always, isolated from broilers when *Campylobacter* spp. are present. Of 28 carcasses tested, 64% were positive for both species, 10.7% for *Campylobacter* spp. only and 10.7% for *Arcobacter* spp. only; 14.3% were negative. There were skips in isolations from consecutive whole carcass rinses, indicating low levels of *Arcobacter* spp. on broilers and/or sporadic recovery. The association of such isolations from poultry carcasses and human disease is still unproven.

OBJECTIVE D:

To develop a defined competitive exclusion (CE) culture by using specific criteria to select microflora from the chicken intestinal tract inhibitory to *Salmonella typhimurium*.

PROGRESS D:

Growth in intestinal mucus is necessary for the successful colonization of the chicken intestinal tract by *Salmonella*. Phosphatidylserine (PS) has been reported in the literature as a component of intestinal mucus that is an important nutrient for *Salmonella* growth. Results in our laboratory demonstrated decreased in vitro growth of *S. typhimurium* in isolated chicken mucus after treatment with phospholipase. This is consistent with a role for PS (or other phospholipids) in supporting growth of *S. typhimurium*. The experimental strain of *S. typhimurium* and 89% of intestinal bacterial isolates of the family *Enterobacteriaceae* could

use PS as sole carbon and nitrogen source (PS+). These strains grew as well as *S. typhimurium* in chicken mucus. They reduced growth of *S. typhimurium* when grown in mixed cultures. When given orally to young chicks before they were given *S. typhimurium*, a mixture of PS+ strains of *Enterobacteriaceae* often, but not always, decreased subsequent colonization of the ceca by the experimental strains of *S. typhimurium*. These strains are likely to be useful components of a defined CE culture. Future plans involve more clearly defining the role of PS in *Salmonella* growth in mucus and isolating additional intestinal strains of bacteria that inhibit growth.

PUBLICATIONS:

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MICROBIAL SAFETY CRITERIA FOR FOODS CONTACTING REUSE WATER IN FOOD PLANTS

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CRIS #: 1935-42000-023
FSIS #: I-94-9
Completion date July 1, 1997

OBJECTIVE A:

To identify means to prevent microbiological hazards that are barriers to the increased utilization of reuse water during slaughter and further processing and acquire data needed to establish safety criteria.

PROGRESS A:

Growth potential of bacterial pathogens in reconditioned water from Hatfield Quality Meats is being assessed. Several experiments were performed comparing the response of *Aeromonas hydrophila* and *Salmonella* that were inoculated into potable, non-chlorinated reconditioned water, and chlorine-treated reconditioned water. Chlorinated water was effective in causing a decline in these organisms. Nutrient status of the medium prior to water-exposure is undergoing evaluation to determine bacterial pathogen growth potential in thiosulfate-treated or unchlorinated water. This is important because preliminary studies demonstrated that residual nutrients from the diluting medium spuriously permitted growth in non-chlorinated water.

OBJECTIVE B:

To study bacterial attachment mechanisms and develop approaches to dislodge or prevent adhesion of pathogens to food and processing surfaces.

PROGRESS B:

A preliminary in-plant comparison of bacterial microflora from carcass surfaces and the water effluent stream of a post-evisceration carcass washer was performed to assess bacterial attachment onto pork carcasses. Generally, there was little correlation between the water and skin surfaces with regard to total aerobic and coliform counts. Work will continue to determine an appropriate method to relate carcass surface with wash water bacterial flora.

OBJECTIVE C:
To investigate new methods to determine food product safety, water safety, and sanitation quality.

PROGRESS C:

An F+RNA coliphage assay was used to assess the efficacy of the Hatfield water reconditioning system. About a 5 $\log_{10}$ reduction was observed when samples were assayed throughout the water treatment. Chlorination had the greatest phagocidal effect. There was a high correlation between total coliforms and coliphage levels ($r^2 = 0.85$). Current and future research will be directed to further assess this potential indicator organism within the slaughter and processing environment.

OBJECTIVE D:
To study the potential for expanded applications of reuse water in the food plant environment.

PROGRESS D:

a) Truck Washing:
An evaluation was started to determine the potential for using reconditioned water for the initial washing of swine trucks between hauls. Field studies were completed for spring and summer seasons. Swab samples were obtained from a statistically-based sampling scheme from truck floors before and after washing. Bacterial analyses were performed to quantitatively determine total aerobic counts, total coliforms, biotype I *E. coli*, and *Salmonella*. Data indicated that all prewashed truck floors had confirmed levels of *Salmonella* that were reduced to below the detectable level after washing. The study is expected to continue into 1995 and will assess hauling distance and seasonal effects.

b) Solution Reuse:
The adequacy of Meat Poultry Inspection Bulletin 83-16 was assessed by a model system experiment that simulated a brine chiller. Pathogens studied included *Listeria monocytogenes*, *Staphylococcus aureus*, and *Yersinia enterocolitica*. Environmental conditions included salt levels of 0-20%, temperatures of -12 to 28°C, and incubation duration of 0-30 d. Results indicated that the brine, temperature, and holding time requirements in the bulletin are adequate to prevent growth of these bacterial pathogens. A parallel study was initiated to determine how propylene glycol (PG) affected the growth of these organisms. Conditions, including water activity levels, are identical to the brine experiment. The data collection for this study is expected to be completed by January 1995.

PUBLICATIONS:

Miller, A.J., T. Brown and J.E. Call. Comparison of bacterial adherence to wooden and plastic cutting boards. J. Food Protect. (Submitted).

SCREENING TEST FOR HAPTOGLOBIN

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CRIS #: 6202-32000-006
FSIS #: I-92-5
Completion date March 11, 1996

OBJECTIVE A:

Develop an immunological-based test for screening cattle at slaughter for the presence of the acute phase reactant, haptoglobin.

PROGRESS A:

An immunoassay has been developed to detect the presence of haptoglobin, an acute phase reactant, in the serum of cattle. Detection of acute-phase reactants such as haptoglobin have been identified by FSIS as a priority item in the development of better methods for antemortem evaluation of beef cattle prior to slaughter. We have generated a monoclonal antibody to haptoglobin following immunization with a partially purified immunogen. Hemoglobin binding to the haptoglobin does not interfere with binding of our monoclonal antibodies. Three different immunoassays for determining levels of haptoglobin in bovine serum samples have been developed and evaluated. These three methods are (i) direct sera binding in microtiter plates, (ii) a direct hemoglobin binding immunoassay, and (iii) a haptoglobin competitive inhibition immunoassay. These methods have been verified in a collaborative study with an FSIS scientist (Dr. P.K. Saini) using slaughterhouse serum samples. Using these immunoassays, we have assayed over 2000 bovine samples involving some 30,000 assays. We have determined that the most reliable and reproducible immunoassay is the Direct Hemoglobin Binding ELISA. The study is currently being prepared for publication. In addition, bovine samples have been assayed from a variety of inflammatory diseases and the following conclusions have been obtained:

(a) Haptoglobin levels are raised in calves with Icteric fever. Study done in collaboration with Dr. Mary Gray, University of Georgia, Athens, and USDA-FSIS.

(b) Haptoglobin levels are raised in cattle with Lyme disease or Johne's disease. Study done in collaboration with Dr. Tom Collins, University of Wisconsin, Madison, WI.

(c) Haptoglobin levels are raised in cattle with Milk fever. Study done in collaboration with Dr. Jesse Goff, USDA-ARS-Northern Plains Area, Ames, IA.

(d) Haptoglobin levels are raised in cattle with shipping fever, and are subsequently reduced following treatment. Study in collaboration with Drs. Travis Littledike and Tom Wittum, USDA-ARS, Clay Center, NE.

(e) Cattle initially having elevated levels of bovine haptoglobin show reductions to normal levels following either antibiotic therapy or antibody administration. Study in collaboration with Drs. Travis Littledike and Tom Wittum, USDA-ARS, Clay Center, NE.

PUBLICATIONS:

Sheffield, C.L., Stanker, L.H., Holtzapple, C.K., and DeLoach, J.R. 1994. Production and characterization of a monoclonal antibody against bovine haptoglobin and its use in an ELISA. Vet. Immunol. Immunopath. 42:171-783.

SPECTRAL RADIOMETRY AS AN ON-LINE INSPECTION TOOL FOR POULTRY

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CRIS #: 1280-42000-008
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Completion date January 1995

OBJECTIVES:

The objective of this project is to develop a real-time, efficient system capable of detecting abnormal poultry carcasses, based on their spectral characteristics in the ultraviolet, visible, and near-infrared regions. The ultimate goal is to incorporate the system into inspection lines in poultry processing plants.

PROGRESS:

The ISL has successfully assembled a transportable NIR spectrophotometer system and obtained reflectance spectra of poultry carcasses on-site at slaughter plants in West Virginia and Maryland's Eastern Shore. Because small sample numbers of the bruised, tumorous, air-sacculitis, and ascitic carcasses were tested, the development of classifiers was restricted to normal, septicemic, and cadaver carcasses. The results showed that the visible/NIR spectrophotometer system can effectively separate carcasses into normal, septicemia, and cadaver, or into two classes, normal carcasses from abnormal (septicemic and cadaver carcasses) with very high accuracies (average accuracies, 90.6 and 96.4%, respectively). The classifier was then implemented into the system, which is able to scan and classify each carcass in real-time. The classifier can be updated or retrained to adapt to any new or changed environment right at the slaughter plants.

Preliminary results of the multi-spectral imaging system showed that it is also capable of discriminating normal poultry carcasses from abnormal (septicemic and cadaver) carcasses with very high accuracy. Based on textural analyses of limited multi-spectral images of carcasses, it was found that images at 542 nm wavelength provided best results for discrimination of the normal and abnormal carcasses with very high accuracy. Perfect separations of the normal and abnormal carcasses were achieved, when a neural net model was used. A transportable multi-spectral imaging system for on-site acquisition of carcass images at slaughter plants was then assembled.

Our results proved the technical feasibility of using visible/NIR spectroscopic and imaging techniques for separation of diseased and cadaver carcasses from the wholesome carcasses. The visible/NIR spectroscopic technique is a rapid method; however, classification with an imaging system, based on textural characteristics of image surface, requires a substantial amount of time for processing and it could not be implemented on-line at the present time.

Fast, efficient algorithms for classification are needed for the system to be able to conduct classification in a real-time fashion.

Rapid methods for identification of bacteria, including immunofluorescence, mass spectrometry, flow cytometry, and resonance Raman methods, were reviewed. Dr. Wilfred Nelson, et al., of Rhode Island University were contracted to conduct a preliminary study on the feasibility of rapid detection of *E. coli* in meat with UV resonance Raman method. They found that differentiation of bacteria at the genius level exists, and in some cases differentiation at the species level may exist. Spectra of bacteria as few as 25 could be obtained with a collection time as short as 5 seconds. Results suggest that identification of bacteria located on the surface of meat may be possible using the resonance Raman method with a CCD detector and a CW laser which produces excitation at the wavelengths which provide the best differentiation between species.

This project will be rewritten for 3 more years funding. During next 3 years, visible/NIR spectral data and multi-spectral images of the viscera, as well as inside and outside surface of normal, septicemia, cadaver, bruised, tumorous, air-sacculitis, and ascitic carcasses will be collected on-site at various slaughter plants for ISL to establish a complete data base. Effective, robust classifiers for classification of the carcasses will be developed and implemented into the transportable system.

Efficient algorithms to detect unwholesome carcasses with the transportable imaging system for real-time application will be developed. Economically feasible methods to implement both spectroscopic and multi-spectral imaging systems on-line at slaughter plants will be studied.

PUBLICATIONS:

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Chen, Y.R., R.W. Huffman, and B. Park. 1993. Visible/NIR spectrophotometer for monitoring shelf life of chicken carcasses. ASAE Paper No. 936067. Presented at the 1993 ASAE Summer Meeting, Spokane, Washington. (Abstract).

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MICROBIAL MODELING

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FSIS #: I-91-7
Completion Date: September 30, 1995

I. Growth Kinetics Modeling

OBJECTIVE A:

Assess the feasibility of developing a means of modeling the impact of microbial competition on the growth of foodborne pathogens in food products.

PROGRESS A:

The effect of microbial competition on the growth of *Listeria monocytogenes* Scott A is being evaluated by determining its growth kinetics when grown in co-culture with one of four other psychrotrophic bacteria, *Carnobacterium piscicola* LK5, *Carnobacterium piscicola* 2562, *Aeromonas hydrophila*, and *Pseudomonas fluorescens*. Environmental variables being considered include incubation temperature (4, 12, & 19°C), initial pH (5, 6, & 7), and sodium chloride concentration (0.5, 2.5, and 4.5%). The two *C. piscicola* strains were selected as representing bacteriocin-producing (strain LK5) and bacteriocin-negative (2562) isolates that would compete with *L. monocytogenes* in refrigerated meat. Data acquisition is complete with both of these microorganisms; however, the results observed with strain 2562 prompted a reevaluation of its ability to produce a bacteriocin. After an exhaustive evaluation of the strain, it was ultimately concluded that it produces an apparent membrane-bound bacteriocin that is not detected in the normal screening procedure. The two strains of *C. piscicola* had a similar effect on *L. monocytogenes*. The primary impact on the pathogen was a suppression of the maximum population density (MPD). Generally, lag phase durations and generation times were not affected. Initial analysis of the data indicates that the extent of MPD suppression is a function of the two species' relative growth rates. Inhibition of *L. monocytogenes* is associated with *C. piscicola* achieving stationery growth. Conditions that favored the relative growth of *C. piscicola* (e.g., low temperature + low sodium chloride) strongly suppressed *L. monocytogenes*' MPD. Conversely, conditions that favored the relative growth rate of *L. monocytogenes* (e.g., high temperature + high sodium chloride) evidenced little depression of the pathogen's MPD.

Different options for modeling the effects observed are currently being investigated. Co-culture experiments with A. *hydrophila* have been initiated, and will be followed by studies with *P. fluorescens*. Once completed, this project should provide a systematic

assessment of the relative importance of microbial competition in controlling the growth of pathogenic bacteria in foods.

OBJECTIVE B:

Assess the ability of growth kinetic models based on the use of sodium chloride for modification of water activity (a_w) to accurately predict the growth kinetics of pathogenic foodborne bacteria in food systems using other humectants.

PROGRESS B:

Humectant Identity:

The effect of humectant identity on bacterial growth kinetics is being studied using *Escherichia coli* O157:H7, a foodborne pathogen for which growth kinetics models have been successfully developed using sodium chloride as the humectant. Humectants being evaluated include mannitol (50, 100, 150, & 200 g/L), sorbitol (50, 100, 150, & 200 g/L), and sucrose (50, 100, 200, & 300 g/L). Other variables being considered are incubation temperature (12, 19, & 28°C) and initial pH (4.5, 5.5, 6.5, & 7.5). Data acquisition for mannitol and sorbitol is complete. Approximately half of the data needed for sucrose have been generated, and this will be completed during the next six months. Initial analysis of the sorbitol and mannitol data suggest that while distinct differences in the response of *E. coli* O157:H7 can be attributed to humectant identity, these differences are largely limited to the lower a_w levels. At higher a_w levels (a_w greater than or equal to 0.973), the growth kinetics of the pathogen could be reasonably predicted using the previously developed, NaCl-based growth kinetics models. These data are currently being more rigorously evaluated to assess the extent of deviation at the lower a_w levels. Likewise, the data will be used to assess the potential for alternate modeling approaches that could more effectively account for humectant identity effects in semi-dry foods.

Sequestrants:

Collection of data on the effect of a long chain sodium polyphosphate, Hexaphos (average chain length: P = 13), on aerobic growth of *L. monocytogenes* Scott A has been completed and is being statistically analyzed and organized for development into a growth model. Growth studies were done using BHI broth to quantify the effects of polyphosphate concentration in combination with temperature (4 - 19°C), pH (5 - 7) and sodium chloride (0.5 - 4.5%). The bacteriostatic effect of the polyphosphate was increased by low pH, low temperature and high sodium chloride concentration.

OBJECTIVE C:

Develop effective growth kinetics models for the growth of *Listeria monocytogenes* in meat and poultry products.

PROGRESS C:

Since the introduction of the original growth kinetics models for *L. monocytogenes* in 1990, our laboratory has continued to accumulate data on the pathogen's growth characteristics. The data set currently includes approximately 1100 individual growth curves. These data have been submitted for the development of expanded growth kinetics models. These models should be available in approximately three - four months.

OBJECTIVE D:

Develop a set of expanded response surface models for *Yersinia enterocolitica* that extend the effective temperature range to include abusive temperatures (19 - 42°C).

PROGRESS D:

The previously reported data set for the low temperature growth (5, 12 and 19°C) of *Yersinia enterocolitica* was expanded to include higher abusive temperatures (28, 37 and 42°C). In addition to temperature, variables considered included the effects and interactions of initial pH (4.5 to 8.5), sodium chloride (0.5 to 5.0%), and sodium nitrite (0 - 200 ug/ml). The data set was used to develop response surface models which proved to be highly effective. These expanded models will be included in the next version of the Pathogen Modeling Program.

OBJECTIVE E:

Develop an expanded model for the aerobic growth kinetics for *Aeromonas hydrophila*.

PROGRESS E:

Recent experiments with *Aeromonas hydrophila* yielded 102 additional growth curves representing 34 additional unique variable combinations for *A. hydrophila*. These data were appended onto the initial aerobic database of 166 variable combinations. The expanded database was analyzed to generate response surface models using various transformations of both the Gompertz B & M terms and the derived values for generation time (GT) and lag phase duration (LPD). The derived equations were also subjected to backward elimination regression analysis to produce simplified versions (i.e., ones containing fewer terms) of the equations. All equations generated were analyzed for their predictive capacity, with the full cubic models of LN-transformation of GT and LPD selected as the one most effective for predicting the growth of *A. hydrophila* at various culture conditions. Comparison of our model to published models for this bacterium suggests that the *A. hydrophila* complex may be too varied genotypically to have a single model which is applicable for all strains.

OBJECTIVE F:

Develop and validate models for the growth of *Shigella flexneri* in foods.

PROGRESS F:

An updated response surface model for the effects of temperature (12 - 37° C), pH (5.5 - 7.5), sodium chloride (0.5 - 4.0%) and sodium nitrite (0 - 1000 ppm) on anaerobic growth of *Shigella flexneri* in BHI broth has been obtained, validated and included into the newest version of the MFS Pathogen Modeling Program.

"Inoculated pack studies" were initiated with *S. flexneri* as a means of validating of available growth kinetics models. Commercially available sterile foods (milk, beef broth, vegetable broth, meats, seafood, vegetables) were inoculated with *S. flexneri* 5384 and incubated at 12, 15, 19, 28 or 37°C. Growth curves were obtained and generation times and lag times calculated. The observed kinetics values were compared with values predicted using growth models based on measurements or estimations of the foods' temperature, pH, NaCl and $NaNO_2$ levels. Observed and predicted values compared favorably for growth in the temperature range of 19 - 37°C. At 12 and 15°C the observed growth rates were generally faster than that predicted by the models. The discrepancy appears to be more active growth after a greatly extended lag phase that was not sufficiently taken into account in the initial aerobic models for *S. flexneri*. Additional growth studies are being carried out in BHI in the temperature range from 10 - 19°C to improve the accuracy of the growth models at lower temperatures.

OBJECTIVE G:

Assess the ability of predictive models developed under isothermal conditions to accurately estimate the behavior of pathogenic bacteria under non-isothermal conditions that would be encountered with foods.

PROGRESS G:

The effect of fluctuating temperatures on bacterial growth kinetics is being studied using *Escherichia coli* O157:H7, a foodborne pathogen for which growth kinetics models have been successfully developed based on static temperatures. A simple six-hour square-wave temperature cycle was used to study transitions between 4 - 12, 4 - 19, 4 - 28, 8 - 19, and 12 - 28°C. In addition, static temperatures of 8, 10, 12, 19, and 28° C were also studied. Other variables considered included initial pH (5, 6, and 7) and sodium chloride level (0.5, 1, 2, and 3%). Initial data acquisition is complete. Growth was observed at 8°C after extended incubation of greater than 500 h. Initial analysis of the data for the fluctuating temperatures suggests that the microorganism responded to pH and NaCl in a manner similar to that observed with the static temperature cultures. Step-like growth curves were observed and were more pronounced for growth with 2 and 3% NaCl. Growth curves were fitted

using the Gompertz equation and compared to the growth parameters obtained using the static growth kinetics models. Initial analysis indicated that the fluctuating temperatures kinetics were more closely approximated by the higher temperature than the mid-point temperature of each cycle. These data are currently being evaluated for development of growth kinetics models, which should be completed in four - five months.

II. Non-Thermal Inactivation Modeling

OBJECTIVE A:

Develop an effective model for predicting the inactivation of *Listeria monocytogenes* when placed in an adverse acidic environment of the type that might be encountered in fermented and non-fermented meat products.

PROGRESS A:

Response surface techniques were used to generate four-variable (temperature, 4 - 42°C; lactic acid, 0 - 2%; pH, 3.3 - 7.3; NaCl, 0.5 - 19.0%; and $NaNO_2$, 0 - 200ug/ml) models for the time to achieve a 4-D inactivation (T4D) of *L. monocytogenes* under conditions of restricted oxygen content. Two sets of models were developed; one for temperature X NaCl X $NaNO_2$ X lactic acid content and the other for temperature X NaCl X $NaNO_2$ X pH. The latter was deemed as more effective and has been incorporated, along with the corresponding aerobic model, in the latest release of the MFS Pathogen Modeling Program. Comparison of the aerobic and anaerobic data suggests that oxygen content has little, if any, effect on the non-thermal inactivation of *L. monocytogenes*.

It was concluded after development of the four-variable models that because foods are often highly buffered, the effects of lactic acid concentration and pH had to be differentiated for the model to be fully effective. Accordingly, inactivation curves representing approximately 35 additional variable combinations for both aerobic and anaerobic conditions were generated where the pH was modified after the addition of lactic acid. These data were appended unto existing data sets and five-variable models (temperature X NaCl X $NaNO_2$ X lactic acid X pH) have been generated for both aerobic and anaerobic conditions. Initial assessment of the models is very encouraging, and it is anticipated that these models should be completed in three - four months. These expanded data sets will also allow us to ask more mechanistic modeling questions such as the effectiveness of using undissociated concentrations for lactic acid and $NaNO_2$.

A final analysis was completed of data from an evaluation of the inactivation of *L. monocytogenes* in the presence of two combinations of organic acids. The two combinations, acetic acid (90% w/w) + EDTA (8%) + ascorbic acid (2%) and propionic acid (90%) + EDTA (8%) + ascorbic acid (2%), accelerated the rate of inactivation in mildly acidic conditions (pH 4.0 - 4.5), particularly at refrigeration temperatures. At lower pH levels, the pH alone was the primary factor controlling inactivation rates. The effects of the individual

components of the mixtures were largely additive. The data suggest that such mixtures could increase the inactivation of pathogens in acidified meat products and that mixtures of acids could be used to avoid organoleptic problems associated with the use of high concentrations of a single acid.

Future research will determine how the physiological state of the cells affect survival. Growth versus stationary phase, temperature, pH, and starvation are factors that may be important.

OBJECTIVE B:

To develop a model to predict the *Staphylococcus aureus* survival during storage of foods not given a thermal process.

PROGRESS B:

Survival in BHI broths made with various pH values (3-7), NaCl levels (0.5-20%), lactic acid (0-1%), $NaNO_2$ (0-200 ppm) and stored at 4 to 42°C was determined. Data were collected for all of the 159 treatment combinations, the primary model was fitted, and the final regression equations and confidence intervals were calculated. This model was incorporated into Pathogen Modeling Program version 4.0. The results showed rapid inactivations at pH less than or equal to 5. Inactivation rates were low at low temperatures and the rate increased with increasing nitrite and lactate concentrations. Salt had relatively little effect when less than or equal to 8%.

OBJECTIVE C:

To develop a model to predict the survival of *Salmonella* during storage of foods not given a thermal process.

PROGRESS C:

The survival data in 163 BHI broths having combinations of environmental conditions was collected for up to 4 mo. The factors and their ranges were temperature (5-42°C), pH (3.5-7.2) [lactic acid 0-1.5%], NaCl (0.5-16%), and $NaNO_2$ (0-200 ppm). Statistical analysis and model development were completed, including calculation of confidence ranges. The model was incorporated into the Pathogen Modeling Program version 4.0.

OBJECTIVE D:

To develop models to predict the influence of acid anions on bacterial survival during storage of foods not given a thermal process.

PROGRESS D:

Survival data is being collected to determine the effect of different organic acids and to model the interactive effect of pH and lactate concentration. Preliminary results show that lactic and acetic acids shorten the survival times relative to hydrochloric and citric acids at the same pH value. Similar patterns of survival times in response to temperature, pH, salt, nitrite and lactate are being observed as were observed for the other pathogens. The collection of survival data will continue.

OBJECTIVE E:

To determine whether survival of selected pathogens in commercial foods would be accurately predicted by the inactivation/survival models that were developed in broth cultures.

PROGRESS E:

Cheddar cheese, fresh oysters, pepperoni, beef jerkey and ham were purchased from a supermarket and their pH values, titratable acidities, salt levels, moisture contents, water activities, and residual nitrite levels measured. They were inoculated with *L. monocytogenes* (oysters, pepperoni, ham), *Salmonella* (cheddar cheese, oysters) or *S. aureus* (cheddar cheese, pepperoni, beef jerkey, ham), stored at 5 and/or 19°C, and the survivors determined during storage. Data was collected and preliminary analyses and comparisons to the predictive models previously developed have begun.

OBJECTIVE F:

Demonstrate the potential for using predictive microbiology models to develop enhanced estimates that can be employed to begin performing quantitative microbial risk assessments of food processing and food preparation operations.

PROGRESS F:

A "position" paper was written that developed a conceptual framework on how predictive microbiology techniques could be integrated with microbial risk assessment models to evaluate the risk associated with modifying individual steps in a food processing scheme. Employing hypothetical examples of ready-to-eat foods, sequential models for estimating the impact of individual food processing and preparation steps were used to assess microbial growth and survival. This included considering a distribution of pathogens in the original raw materials. The estimates of pathogen levels were then used to calculate "probabilities of infection" based on published dose-response data. The overall conclusion was that it should be possible to perform rudimentary risk assessments of processed products using currently available data. Future research will focus on refining these concepts and testing them with data acquired for a selected processed meat product.

OBJECTIVE G:

Technology transfer: Develop "user-friendly" software that allows the mathematical models to be readily used by FSIS personnel and the food industry.

PROGRESS G:

A new version of the MFS Pathogen Modeling Program (release 4.0) was completed and is being distributed to individuals on our distribution list. This includes approximately 700 individuals and organizations. We are currently in the final phases of developing a CRADA with a software developer so that the next version will have additional features and will be "Windows compatible."

Negotiations continue on the development of an "international database" for the purposes of generating enhanced models and for commercializing a joint software package. An initial contract has been drafted by the British participants, and is currently being reviewed by the U.S., Canadian, and Australian teams. The U.S. negotiating team has representatives from ARS, FSIS, and FDA. The goal is to have a contract in place and begin initial model development by January 1, 1995.

OBJECTIVE H:

Determine the potential for killing bacterial pathogens with pulsed, high voltage electrical fields and how various environmental factors affect the rate of killing.

PROGRESS H:

E. coli O157:H7 cells were suspended in various broth media and given a series of electrical field pulses (nominal half- life 15 ms, 2.5 kV/cm). In 0.1% peptone, 10 pulses reduced the viable count by 5 logs, in water by 8 logs. The killing rate was greatest in dilute NaCl (0.1%). Dextrose or fructose did not affect the rate until high concentrations (greater than 5%). When the pH was $\leq$ 4 the rate was faster, at pH values $\geq$ 5 there was no pH effect. Temperature had little influence $\leq$ 37°C, at 50 to 60°C the killing rate was accelerated compared to the lower temperatures. *C. sporogenes* spores were not affected by treatment with 20 pulses at ambient temperature. This research demonstrates that high voltage electrical fields can effectively kill vegetative cells under appropriate conditions in broths. Efforts are underway to obtain equipment capable of administering higher strength fields and then testing the effectiveness of this presence in foods.

OBJECTIVE I:

To determine the potential for the bacteria, *Bdellovibrio*, to attack, parasitize and kill gram negative foodborne pathogens.

PROGRESS I:

Bdellovibrio isolates able to attack and reduce populations of *E. coli* serotype O157:H7 and *Salmonella* species were isolated from soil and sewage samples. Using a two-membered culture system, the reduction in the level of the host organisms (*E. coli* and *Salmonella* strains) ranged from 2.5 to 7.9 log values after 7 hr incubation at 30°C. The host organisms were lysed most effectively at temperatures between 25 and 37°C. The *Bdellovibrios* were also able to attack and reduce the level of *E. coli* O157:H7 dried onto stainless steel surfaces. The next year will attempt to demonstrate effective reductions of these pathogens in situations closely simulating food processes.

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GROWTH AND TOXIN PRODUCTION OF HARMFUL PSYCHROTROPHS (INCLUDING *LISTERIA MONOCYTOGENES*)

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FSIS #: I-88-1
Completion date February 24, 1995

OBJECTIVE A:

Development of a PCR method for the isolation and detection of *Escherichia coli* O157:H7.

PROGRESS A:

Studies were continued on the development of a polymerase chain reaction (PCR) procedure for detection of *E. coli* O157:H7. Primers were designed in our laboratory that amplify a sequence of the large plasmid harbored by virtually all *E. coli* O157:H7 strains and recently linked to a new virulence factor, hemolysin production. This primer is being used in combination with primers which amplify sequences of two other *E. coli* O157:H7 virulence genes (shiga toxin and attachment) in a multiplex PCR. The three DNA fragments were successfully amplified in one reaction. The detection limit for amplification of the plasmid fragment is approximately 1 CFU using crude cell lysates. PCR conditions are being optimized to obtain equal amplification of the three DNA fragments in the multiplex PCR. The multiplex PCR was also used for detection of the pathogen in artificially inoculated ground beef. Preliminary results indicate that very low levels of the organism can be detected after a 6 hour enrichment. The sensitivity of the procedure increases approximately 100-fold when the *E. coli* are first recovered from the ground beef enrichment cultures using magnetic beads coated with antibodies against *E. coli* O157:H7. Rapid and sensitive colorimetric and chemiluminescent methods for detection of the amplified DNA fragments are being developed.

OBJECTIVE B:

Detection of psychrotrophic and other foodborne pathogens.

PROGRESS B:

Low calcium Congo red agarose medium was used as a single multiple test medium to identify pathogenic serotypes of *Yersinia enterocolitica* or to indicate the pathogenic potential of individual strains. The assay is based on Congo red uptake, crystal violet binding, low calcium response and hydrophobicity. In general, the efficiency and reliability of this medium tested was found to be better than existing dye binding techniques for detecting

virulent *Y. enterocolitica* strains. Investigation on the expression of plasmid-encoded cell surface proteins at 28°C is complete. There was no expression of plasmid-encoded cell surface proteins at 28 °C to use for the specific isolation of plasmid-bearing virulent strains of *Y. enterocolitica* by immunomagnetic separation (IMS). At present, immunological characterization of chromosomally expressed surface protein(s) of pathogenic *Y. enterocolitica* is in progress. Since IMS also isolates non-pathogenic plasmidless strains, techniques such as PCR, nucleic acid hybridization and dye binding will be evaluated for detection of plasmid-bearing virulent strains of *Y. enterocolitica*. The specificity of surface protein antibody(s) generated by serotype O:3 (GER strain) will be evaluated for the isolation of other serotypes of *Y. enterocolitica* by IMS. Computer analysis of the sequences of heat labile enterotoxin gene(s) for their potential use in the development of universal probe(s) for identification of various foodborne bacteria is in progress.

OBJECTIVE C:

Attachment and detachment of *E. coli* O157:H7 from beef tissue.

PROGRESS C:

Studies were continued on the attachment of *E. coli* O157:H7 to beef. Selected rinsing agents (trisodium phosphate [TSP] and acetic acid) used alone and in combination were compared for their efficacy in reducing the number of viable *E. coli* attached to beef tissues. Two rinses with TSP (10%, pH 12.4) was the most effective rinse combination in reducing the number of *E. coli*, but the effect was restricted to beef adipose tissue. There was a 3.4 log decrease in the level of *E. coli* O157:H7 attached to adipose tissue following a double TSP rinse combination compared to rinses with a buffered saline solution.

OBJECTIVE D:

Determine the minimum and maximum temperature of growth and verotoxin production by O157:H7 and other serotypes of *E. coli*.

PROGRESS D:

The influence of temperature on growth and verotoxin production by *Escherichia coli* strains was studied in brain heart infusion (BHI) broth in both shake cultures at various temperatures and in a temperature gradient incubator. Both hemorrhagic and non-hemorrhagic strains of *E. coli* grew from at least 10°C to 45°C, with some strains growing at 8°C. Verotoxin production (determined using the Vero cell assay system) was a function of both temperature and time, with the highest titer produced at temperatures supporting the fastest growth and largest viable cell counts. However, for strains producing verotoxin, toxin production was detected at any temperature supporting growth. Substantial increases in numbers were seen for several strains in 4-6 days at 10°C. The data presented here indicate that while *E. coli*

may not be considered a true psychrotroph, most strains can easily grow at ca 10°C and thus increase readily and produce toxin during temperature abuse.

OBJECTIVE E:

To identify factors for controlling of the psychrotrophic pathogen *Aeromonas hydrophila.*

PROGRESS E:

Previous work indicated that the growth of *A. hydrophila* could be controlled by a combination of polyphosphate and NaCl. This initial observation was extended with the finding that the combination of 2% polyphosphate (tripolyphosphate, sodium pyrophosphate, sodaphos, or hexaphos) and 3-1/2% NaCl was lethal to the bacterium. D-values were determined at temperatures from 5 to 42° C. Using a tryptic soy agar (TSA) and TSA + 2% NaCl (TSAS) plating system, the polyphosphate-NaCl combination was found to injure the cells. Electron microscopy of cells treated with the combination of polyphosphate-NaCl revealed that the cells showed both surface and cytoplasmic changes and ultimately the cells were completely destroyed. This effect was also evaluated in several food systems. In naturally contaminated scallops that were dipped for two minutes in a solution of 2% polyphosphate and 3.5% NaCl; there was about one log cycle decrease in *A. hydrophila* at the time of treatment, but this small effect was lost on storage of the treated scallops at 5°C. The addition of polyphosphate-NaCl to ground pork inoculated with *A. hydrophila* yielded a decrease in numbers of the bacterium at the time of addition, and continued suppression of *A. hydrophila* on storage at 5°C. The effectiveness of the combination in the ground pork system versus the scallop system likely reflects contact time. The scallops had only a two minute exposure to the combination while with the ground pork, the bacterium was continuously exposed to it. These results suggest that polyphosphates could be useful to control the presence of *A. hydrophila* in certain foods.

OBJECTIVE F:

To determine the potential for controlling *Listeria monocytogenes* by combinations of polyphosphate, pH, NaCl and temperature.

PROGRESS F:

Studies on the growth inhibition of L. monocytogenes by sequestrants (polyphosphates, EDTA, citrate) are continuing. Studies on the effect of a long chain sodium polyphosphate, Hexaphos (average chain length = 13), in combination with temperature (4, 12, 19 °C), NaCl (0.5, 2.5, 4.5%) and initial pH (5, 6, 7) on aerobic growth of *L. monocytogenes* Scott A have been completed. Increasing concentrations of Hexaphos (0.1 to 1.0%) in Brain Heart Infusion medium progressively delayed bacterial growth. Growth inhibition by the polyphosphate increased with decreasing temperature, decreasing pH and increasing NaCl concentration. Low pH particularly enhanced the inhibitory effect of Hexaphos.

Since sodium polyphosphates are chelating agents, it is necessary to assess the effect of added metal ions on growth of *L. monocytogenes* in media containing polyphosphates. Addition of magnesium, calcium, zinc, manganese or iron (III) ions to BHI media containing 0.5% Hexaphos reversed the growth inhibition by the polyphosphate. Addition of 0.002 M Mn^{+2} or Zn^{+2} eliminated most of the growth inhibition, while higher concentrations were necessary for Fe^{+3} (0.005 M), Ca^{+2} (0.01 M) or Mg^{+2} (0.01 M). These results suggest that the effectiveness of sodium polyphosphates as inhibitors of *Listeria* may be diminished in foods containing high levels of polyvalent metal ions, either naturally present or added as dietary supplements.

Studies have been initiated on the effect of EDTA on the growth of *L. monocytogenes*. Also, combinations of polyphosphates with EDTA will be tested for enhanced inhibitory effect.

OBJECTIVE G:

To compile a database containing references about *Aeromonas*, particularly as related to foods.

PROGRESS G:

An 800+ reprint file has been established for *Aeromonas*. Databases for modeling, *Bacillus cereus*, *Yersinia enterocolitica*, *Clostridium botulinum*, *Helicobacter pylori*, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 are also available on request.

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HEAT RESISTANT *CLOSTRIDIUM BOTULINUM* SPORES IMPACT OF CHANGING TECHNOLOGIES

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CRIS #: 1935-42000-020
FSIS #: I-88-4, I-83-58
Completion date: February 19, 1996

I. CLOSTRIDIUM BOTULINUM

OBJECTIVE A:

Define the heat treatment required to achieve a specified lethality for non-proteolytic *Clostridium botulinum* type B and type E spores in turkey to ensure that the heating step is lethal, while avoiding heating that negatively impacts product quality. Use data to develop a mathematical model for determining the effects of environmental parameters on thermal resistance of *C. botulinum*.

PROGRESS A:

Initial data were collected on the heat resistance of a mixture of non-proteolytic *C. botulinum* type B and type E spores in turkey slurry. Environmental parameters assessed were pH (5.0 - 6.5), NaCl (0 - 3%), and sodium pyrophosphate (0 - 0.3%). Survivors were determined using RCM+lysozyme, which had been previously determined to be the most effective recovery medium. The samples were also plated on recovery medium that included the same environmental parameters as the heating menstruum. The latter reflected the heated spores' potential to grow out in a specific food (turkey) while the former gives the maximum recovery of heat injured spores. The data were analyzed by second order polynomial equations with temperature, pH, phosphate and NaCl concentration. The D-values were used in an untransformed state as well as the natural logarithm. The logarithmic equation yielded an R squared value of 0.828 (adjusted to 0.914) while the untransformed D-values yielded an R squared value of 0.876 (adjusted to 0.930). The R squared values were 0.825 and 0.924, respectively, when the data (D-values) used were obtained using the same conditions in the recovery medium as the heating menstruum. Confidence intervals were developed to allow microbiologists to know the expected resistance of non-proteolytic *C. botulinum* spores in turkey. The model will be incorporated in the next version of the pathogen modeling software.

OBJECTIVE B:

Develop a time-to-turbidity model for a mixture of nonproteolytic *C. botulinum* type B strain spores.

PROGRESS B:

The model estimates the probability (0 to 100%) of a specified number of spores (log number 1 - 5) to grow out over a 90 day period. The environmental factors for the model are temperature (5 - 28°C), pH (5 - 7) and NaCl level (0 - 4%). The model was developed fitting the data on the percentage of positive samples to a logistic primary model. Then polynomial regression equations were calculated for the primary model's parameters. The modeling system has been included in version 4.0 of the Pathogen Modeling Program. The number of spores in the sample had a major impact on the observed time to turbidity for the nonproteolytic model. Because spore numbers proved to be an important factor, additional data collecting was initiated to expand the previously published model for proteolytic A and B strains to include spore levels. When finished, the model should be much more relevant to actual food products than previous modeling and nonmodeling research that used broths or foods inoculated with high numbers (1000-10000) of spores. In addition, the previously published time-to-turbidity model for proteolytic strains was revised to provide confidence intervals. This revised model has been included in version 4.0 of the Pathogen Modeling Program and completes this portion of the CRIS.

OBJECTIVE C:

Identify naturally occurring flavor compounds and raw vegetable extracts, approved for food use, that are inhibitory against *C. botulinum* and other foodborne bacterial pathogens and define their potential as antimicrobial food additives.

PROGRESS C:

Diacetyl (DI), benzaldehyde (BE), pyruvaldehyde (PY) and piperonal (PI) were tested for activity against several foodborne pathogens. Of the compounds tested, 0.78 mM DI or PY was the lowest minimal inhibitory concentration (MIC) observed for *Yersinia enterocolitica*, *Listeria monocytogenes* and *Escherichia coli* O157:H7. Approximately 2 mM DI or PY were required, however, to inhibit *Staphylococcus aureus*, while 0.39 mM DI was active against *Salmonella typhimurium* and *Shigella flexneri*. Although 25 mM DI inhibited proteolytic *Clostridium botulinum* vegetative cells, as little as 0.05 mM DI inhibited *Bacillus cereus* vegetative cells. In general, aliphatics were more active against Gram-negative bacteria, and DI or PY the most effective of the compounds tested. A 30 min-56.8°C heat treatment, reduced the MICs of BE and PI by 50-87%, and 77% less PY was required to inhibit *E. coli* 0157:H7. BE, CI or PI reduced *C. botulinum* spore 80°C thermal- and radio-resistance. To determine if antibacterial plant components exist naturally at inhibitory concentrations, EtOH-extracts of asparagus (AS), carrots (CA), radishes (RA), shallots (SH), and turnips (TU) were tested for antibotulinal activity. AS or CA extracts were antigerminative at 0.07%. When equal mixtures of AS and CA were tested, less than or equal to 0.03% delayed germination. Although no activity was observed when RA and SH were test singly, 0.13% AS+CA+ RA, AS+CA+RA+SH or AS+CA+RA+SH+TU

combinations were inhibitory. Several food manufacturers have expressed an interest in the antibacterial properties of the flavor carbonyls and the vegetable extracts tested.

OBJECTIVE D:

Test phenylacetaldehyde (PH), benzaldehyde (BE), cinnamaldehyde (CI) and piperonal (PI), singly and in various combinations, to determine their effect of 90°C thermal resistance of proteolytic *C. botulinum* spores.

PROGRESS D:

In previous investigations, various approved aromatic aldehydes and ketones reduced *C. botulinum* spore thermal resistance at processing temperatures that normally do not affect the pathogen. Spore thermal resistance was modulated in varying degrees by the flavor compounds tested. At 100 mM concentrations, 90°C thermal death values (4 $\log_{10}$ CFU/ml reductions of 16, 10, 9 and 12 min were observed, respectively, for PH, BE, CI and PI. When tested at 50 mM, significantly longer treatment times were required for each of the compounds: 28, 73, 31 and 163 min for PH, BE, CI and PI, respectively. Additive or synergistic effects were observed when various combinations of the aromatic aldehydes were tested. At a total carbonyl concentration of 100 mM, values of 36, 14, 26 14, 15 and 22 min were required to achieve a 4 $\log_{10}$ CFU/ml reduction in population density for PH+BE, PH+CI, PH+PI, BE+CI, BE+PI and CI+PI supplemented treatments, respectively. Similar botulinal thermal resistance modulations occurred in the presence of 3- and 4-aldehyde combinations. These data indicated that certain approved aromatic flavor compounds may provide a means of reducing the thermal resistance of *C. botulinum* spores.

II. Staphylococcus aureus

OBJECTIVE A:

Evaluate the ability of acetanisole (4'-methoxyacetophenone) (AC), benzaldehyde (BE), cinnamaldehyde (CI), diacetyl (DI), phenylpropionaldehyde (PH), and pyruvaldehyde (PY) to inhibit or control *S. aureus* NRRL B124 in Brain Heart Infusion broth. Variables included evaluating the presence and absence of oxygen, at temperatures 12, 19 and 37°C, and effect of using in combination with mild (20 min at 50 or 60°C) heat treatments.

PROGRESS A:

The carbonyls limited *S. aureus* growth at minimal inhibitory concentrations (MIC) of 0.48-7.81 mM. After 4 h at 37°C, a 2.3 $\log_{10}$ CFU/ml population reduction was observed with cultures containing 7.8 mM PH, PY, DI or CI. Activity was O_2-tension independent, with CI (0.48 mM), DI (1.95 mM) and PY (1.95 mM) being most active. The MIC of CI was temperature independent, while PY was most effective at 19°C, and PH and DI at 12°C. Mild heat treatment of carbonyl-supplemented samples reduced previously observed MICs.

At 60°C, for example, the MICs for AC and BE, 3.90 and 7.81 mM respectively, were both reduced to ≤ 0.48 mM. Carbonyls are effective antistaphylococcal agents and their use in combination with thermal processing may serve as a new approach to control *S. aureus* growth and other Gram-positive foodborne pathogens. As such, the effect of the flavor compounds on *S. aureus* growth dynamics will be tested further in a range of food products to define their utility as antistaphylococcal food additives.

OBJECTIVE B:

Define cellular mechanisms of susceptibility of *Staphylococcus aureus* to the flavor carbonyls phenylpropionaldehyde, pyruvaldehyde, diacetyl (2,3-butanedione), cinnamaldehyde, acetanisole (4'-methoxyacetophenone) and benzaldehyde.

PROGRESS B:

Transitions in *Staphylococcus aureus* WRRC B124 whole cell methanolysate fatty acids were monitored at incubation temperatures previously shown to alter the bacterium's susceptibility to the flavor carbonyls. The range of incubation temperatures tested induced both qualitative and quantitative differences in *S. aureus* whole cell methanolysate fatty acids. Branched chained saturated fatty acids were the predominate species at 37°C, and the aromatic carbonyls were most active at this incubation temperature. At 12 or 19°C, C18:1 or C16:1 fatty acids were the predominate species, and the overall antistaphylococcal activity of the flavor compounds were enhanced. Iso-branched C14:0, C16:0 and C18:0 fatty acids were expressed exclusively at 37°C and several C17:0 and C20:0 fatty acids were suppressed at 12°C. These data suggest that temperature-dependent transitions in cell membrane fatty acids can enhance or antagonize the antistaphylococcal activity of several flavor compounds and as such may provide a means for enhancing inhibitory activity. Additional analyses are currently underway to further define the antibacterial properties of flavor compounds, identify and define optimal inhibitory physical/chemical conditions, and to define the inhibitory mechanism of action of the carbonyl compounds tested.

III. CLOSTRIDIUM PERFRINGENS

OBJECTIVE A:

Develop models for the growth of *Clostridium perfringens* in media and assess the effects and interactions of temperature, pH, sodium chloride, sodium nitrite, sodium lactate, and phosphate.

PROGRESS A:

Experiments were conducted on modeling growth kinetics of a three strain mixture of *C. perfringens* in media. The effect and interaction of temperature, pH, sodium chloride, sodium nitrite, sodium lactate, and phosphate are being assessed. The work is in the data acquisition

phase. Two models, one for spore germination and one for vegetative outgrowth, will be developed in the near future.

OBJECTIVE B:

Develop rapid and sensitive methods for the identification of enterotoxigenic *C. perfringens* in meat.

PROGRESS B:

Molecular biology techniques based on the detection of the enterotoxin gene were used to develop a rapid method for specifically detecting *C. perfringens* type A in beef samples. The first approach involved the development of a digoxigenin-labelled DNA probe for specific detection and direct enumeration of enterotoxigenic *C. perfringens* in raw beef. The procedure combined a membrane filtration procedure with a nonisotopic colony hybridization technique specific for a 364 base pair sequence of the *C. perfringens* enterotoxin gene. After a two hour hybridization with a digoxigenin (DIG)-labelled probe, the membranes were developed using an anti-DIG-alkaline phosphatase conjugated antibody resulting in a chromogenic reaction that enabled detection and enumeration. The technique demonstrated a higher level of sensitivity than conventional cultivation methods while providing evidence for the presence of potentially enterotoxigenic strains of *C. perfringens* as determined by the presence of the enterotoxin A gene. The total detection time was two days with sample processing, membrane filtration, and cultivation on TSC agar the first day, followed by colony hybridization and enumeration on the second day. In the second approach, a Polymerase Chain Reaction (PCR) procedure was developed for direct detection of *C. perfringens* strains with potential for food poisoning in raw beef samples. An oligonucleotide primer pair was used to amplify a 364 base pair sequence internal to the *C. perfringens* enterotoxin gene. The PCR amplification assay developed in this study combined a short enrichment incubation with a chromosomal DNA extraction step preceding amplification. The assay detected the clostridial enterotoxin A gene at levels below 10 CFU/g of meat, in the presence of the background flora ($\sim 10^6$ CFU/g) present in raw beef. Validation of developed techniques will be done and conditions such as radiation treatment and vacuum/low temperature storage will be assessed.

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PATHOGEN CONTROL & VITAMIN RETENTION IN IRRADIATED MEAT

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CRIS #: 1935-42000-025
FSIS #: I-90-1
Completion date November 30, 1996

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215-233-6582

OBJECTIVE A:

Identify common mechanism(s) explaining differences in the sensitivities of foodborne pathogens and vitamins to ionizing radiation in various meats and poultry.

PROGRESS A:

1. Control of enterotoxic *Bacillus cereus* on poultry or red meats, and in beef gravy by gamma irradiation.

We previously reported the results of studies of the radiation resistance of *B. cereus* ATCC 3018 on chicken. This report expands that study to five additional strains of *B. cereus* and to include direct comparisons of the survival of mixtures of the endospores from the six strains under identical conditions on irradiated red meats and poultry. The gamma-radiation resistance of five enterotoxic and one emetic isolate of *B. cereus* vegetative cells and endospores was tested in mechanically deboned chicken meat (MDCM), ground turkey breast, ground beef round, ground pork loin, and beef gravy. The D-10 Values for *B. cereus* ATCC 33018 were 0.184, 0.431, and 2.56 kGy for logarithmic-phase cells, stationary-phase cells, and endospores at 5°C on MDCM, respectively. Neither the presence or absence of air during irradiation, nor irradiation temperature (-20 to +20°C) significantly affected radiation resistance of vegetative cells or endospores of *B. cereus* ATCC 33018. Impedance studies indicated that surviving vegetative cells were severely injured by radiation. A dose of 7.5 kGy at 5°C was required to eliminate a challenge of 4,600 *B. cereus* ATCC 33018 from temperature-abused MDCM (24 h at 30°C). The radiation resistance of a mixture of endospores of six strains to gamma radiation was identical (2.78 kGy) in ground beef round, ground pork loin, and beef gravy, but differed from that found in irradiated turkey and MDCM (1.91 kGy). The results indicate that irradiation of meat or poultry can provide significant protection from vegetative cells but not from endospores of *B. cereus*.

2. Effects of NaCl, sucrose, or water content on the survival of *Salmonella typhimurium* or the retention of thiamin or vitamin E in irradiated pork.

Many processed meat and poultry products have reduced water content, or added salt and sugar to lower the water activity, thereby, restricting the growth of foodborne pathogens. The effect of such treatments on the survival of *S. typhimurium* and of the retention of

thiamin and alpha tocopherol in irradiated pork loin was investigated. Increased retention of thiamin and survival of *S. typhimurium* occurred in irradiated pork containing high amounts of NaCl or reduced amounts of water. Decreased retention of alpha tocopherol occurred in irradiated pork containing reduced amounts of water and NaCl had little effect on the retention of this vitamin. A radiation protective effect for *S. typhimurium* and thiamin was not observed in pork containing elevated sucrose concentrations indicating that the effect was not linked to the water activity of the product. This was confirmed by study of the effects of radiation on *S. typhimurium*, thiamin, and alpha tocopherol in NaCl and sucrose solutions of equal water activity values. The protection afforded by the NaCl solutions was related to their concentration, but this did not occur with the sucrose solutions. This study was conducted primarily to provide basic information about the factors that might affect the response of foodborne pathogens and also vitamins to ionizing radiation, but it also indicates that there may be higher than expected survival of foodborne pathogens in irradiated cured meats especially when the product contains high amounts of NaCl.

3. Radiation resistance of the food spoilage organism *Shewanella putrefaciens* on meat and poultry.

The radiation resistance of the food spoilage organism *Shewanella putrefaciens* was very similar (0.19 kGy) on ground beef, ground pork loin and ground turkey breast. The radiation resistance of this organism has not been reported previously.

4. Radiation resistance of pathogenic *Escherichia coli* serovars on hamburger.

The gamma radiation resistance of two non-pathogenic, two enterotoxic serovars, two enteroinvasive serovars, five isolates of the enterohemorrhagic serovar O26:H11, two isolates of the enterohemorrhagic serovar O111:NM, and four isolates of the enterohemorrhagic serovar O157:H7 were determined at $\pm$ 5°C on hamburger meat. All, with the possible exception of O111:NM, were more resistant to gamma radiation at -5°C than at +5°C. The resistance of these isolates to gamma radiation were very similar, though not necessarily identical, to that of the single isolate of *E. coli* O157:H7 we reported previously.

5. Identification of a stable reaction product of aqueous thiamin and gamma radiation.

The principal product of the reaction of gamma radiation with aqueous thiamin has been identified by HPLC, NMR, and HPLC/MS to be 2-methyl-4-amino-5-aminoethyl-pyrimidine.

6. Cooperative Effects of Hydroxyl Radicals and Oxygen on Radiation-induced Cell Lethality of *Salmonella typhimurium* ATCC 14028.

The lethality of gamma radiation doses for *Salmonella typhimurium* 14028 was measured in the presence of air, nitrogen, nitrous oxide, glycerol, formate, and polyethylene glycol (PEG). Nitrous oxide reacts with the hydrated electron to produce hydroxyl radicals doubling their number, so greater lethality might be expected in its presence. Instead,

saturation with nitrous oxide, nitrogen, and nitrous oxide and nitrogen during irradiation significantly decreased radiation lethality for *S. typhimurium*. Adding 10% oxygen to either nitrogen or nitrous oxide resulted in approximately the same lethality as occurred in the presence of air. Hydroxyl radical scavengers 1 M glycerol, 40 mM formate, and 1.5% w/v PEG-8000 MW provided protection similar to nitrogen or nitrous oxide. Formate provided better protection than the non-permeable PEG-8000. Anaerobically grown cells were more sensitive to radiation, and were less protected by PEG than aerobically grown cells. No radiation induced lipid peroxidation of the cellular components was identified by measuring TBA values. Cytoplasmic extracts protected chromosomal DNA in vitro either in the presence or absence of oxygen. These results indicate that both nitrogen and nitrous oxide act by simple exclusion of oxygen from the irradiated cell suspensions and the cooperative effects of extracellular hydroxyl radicals and oxygen were greater with increased dose.

7. Effect of low dose gamma radiation on lipids in five different meats.

The effects of radiation (up to a maximum absorbed dose of 10 kGy at 5°C) on the lipids of the following meats: *Longissimus dorsi* muscle from pork, lamb, and beef turkey leg meat, and turkey breast muscle were determined under identical conditions. The meats were analyzed immediately following treatment. No effect of irradiation treatment was noted on peroxide or iodine values of the meat lipids. In turkey breast meat, but not in the other meats, a dose related effect on malonaldehyde content was noted. These results indicate that low dose effects of gamma radiation on lipids in these meats are minimal and very similar.

8. Effect of gamma radiation on thiamin and riboflavin in beef, lamb, pork and turkey.

A study was made of the loss of thiamin and riboflavin due to gamma radiation of beef, lamb and pork *Longissimus dorsi*, and turkey breast and leg muscles. Thiamin losses averaged 11 %/kilogray (kGy) and riboflavin losses 2.5 %/kGy above 3 kGy. The rate of loss of thiamin in beef was significantly higher than in lamb, pork, or turkey leg but not turkey breast, with losses of 16 %/kGy in beef and 8 %/kGy in lamb. The rate of thiamin loss was not related to suffhydryl, protein, moisture, fat or water content, pH or the reducing capacity of the meats as measured by redox titration. Radiation induced loss of riboflavin was not different in the five meats and was not detected at 2 kGy or less.

9. Effect of gamma radiation on levels of α-tocopherol in red meats and turkey.

The effect of low dose ionizing radiation on free α-tocopherol levels in beef, pork, and lamb *Longissimus dorsi* muscle and on turkey leg and breast muscle were determined. The samples were irradiated in air with a Cs-137 source at 8 dose levels between 0 and 9.4 kGy at 5°C. Irradiation resulted in a significant decrease in α-tocopherol levels in all meats studied. There were no statistically significant differences in the rates of loss of tocopherol due to species, with the exception of turkey breast meat. The rate of loss of tocopherol in turkey breast tissue was greater than in the other meats. The information obtained in this

study may be of use for "chemiclearance" purposes since the relative effects due to species were examined.

10. Effect of temperature on the radiation sensitivity of *Listeria monocytogenes* on beef.

Longissimus dorsi from beef was inoculated with *Listeria monocytogenes* and the effect of gamma irradiation on the survival of this pathogen at temperatures from -60 to +15°C was determined. Radiation D-values were determined for the inactivation of *L. monocytogenes* at 5°C intervals from -20 to 5°C. These data were used to develop an equation to predict the response of this pathogen to gamma radiation within this range of irradiation temperatures. An abrupt increase in resistance occurred at -5°C. The radiation D-value increased from 0.45 at 0°C to 0.77 and 1.21 kGy at -5 and -20°C, respectively. A straight line was obtained when the $\log_{10}$ of the D-values from -5 to -20°C was plotted against the reciprocal of the absolute temperature, leading the authors to calculate a value analogous to the Arrhenius activition energy for the inactivation of *L. monocytogenes* by gamma radiation.

11. Variations in radiation sensitivity of foodborne pathogens associated with the suspending meat.

Longissimus dorsi from beef, pork, and lamb and turkey breast and leg meats were inoculated with *Escherichia coli* O157:H7, *L. monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*, and the gamma radiation resistance of the pathogens were determined under identical conditions. At a temperature of 5°C the respective radiation D-values of *E. coli* O157:H7 and *L. monocytogenes* were not affected by the suspending meat. The D-value for a mixture of *Salmonella* spp. was significantly lower on pork than on beef, lamb, turkey breast, and turkey leg meats. The D-value for *S. aureus* was significantly lower on lamb and mechanically deboned chicken meat than on the other meats. All values were, nevertheless, within the ranges expected for these pathogens.

12. Killing of different strains of *Toxoplasma gondii* tissue cysts by irradiation under defined conditions.

To study the effect of gamma-irradiation on the viability of *Toxoplasma gondii* tissue cysts, brains of mice inoculated with 95 newly isolated strains of *T. gondii* from pigs and 10 other laboratory isolates were pooled, flattened, packed in vacuum, and irradiated to absorbed doses of 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.9 kGy at 5°C (± 0.5° C) with a Cs-137 gamma-radiation source. Treated samples were bioassayed for viable *T. gondii* in mice, cats, or both. Tissue cysts of all strains were rendered nonviable at 0.4 kGy. To study the effect of temperature on inactivation by irradiation, tissue cysts were irradiated at -4, 0, 4, 8, 12, and 16°C (± 0.5°C) at 0.25 kGy. Temperature during irradiation had no marked effect on the viability of tissue cysts.

The research in this objective is nearly complete, however, industry representatives have requested that data be obtained on the effects of ionizing radiation on pathogens and vitamins

in the "semi-exotic" meats (bison, alligator, and ostrich). This is considered to be a short term research effort. Research is in progress on defining the effects of irradiation temperature on water soluble and fat soluble vitamins and on the identification of their radiolytic products.

OBJECTIVE B:

Evaluate the safety, and efficacy of treating poultry and red meats with ionizing radiation in combination with modified atmosphere or vacuum packaging.

PROGRESS B:

Effects of ionizing radiation and anaerobic refrigerated storage on indigenous microflora, *Salmonella* and *Clostridium botulinum* types A and B in mechanically-deboned chicken were determined. The concept that *Clostridium botulinum* might produce toxin before signs of spoilage was tested using vacuum-canned, commercial, mechanically-deboned chicken meat challenged with *C. botulinum*, types A and B, and *Salmonella enteritidis*. Irradiated (0, 1.5, and 3.0 kGy) samples were stored at 5°C for 0, 2, and 4 weeks. As expected, the highly radiation resistant endospores of types A and B *C. botulinum* survived the treatment. No refrigerated sample was toxic; however, all inoculated samples became toxic when temperature abused. *S. enteritidis* in nonirradiated samples decreased during storage from $\log_{10}$ 3.86 to 2.58. It was countable only in samples irradiated to 1.5 kGy at 0 wk and was not detected by enrichment culture in samples irradiated to 3.0 kGy. Populations of aerobic or facultative mesophiles increased during 4 weeks of refrigerated storage from $\log_{10}$ 6.54 to 8.25, 4.03 to 8.14, and 2.84 to 5.23 in the samples irradiated to 0, 1.5, and 3.0 kGy, respectively. Based on taxonomic analyses of 245 isolates, the microbial populations of samples shifted as a function of both radiation dose and storage time from predominantly gram negative in nonirradiated samples to gram positive streptococci in samples irradiated to 3.0 kGy and stored for 4 weeks. Significant populations of spoilage organisms survived even the 3.0 kGy treatment.

In the future, the effects of ionizing radiation treatments, vacuum and modified atmosphere packaging (mixtures of nitrogen, carbon dioxide, oxygen) on microbial pathogens and vitamin content of turkey breast meat will be investigated.

OBJECTIVE C:

Develop tests that will identify irradiated poultry and red meats.

PROGRESS C:

A simple, rapid and inexpensive method is needed by regulatory agencies to identify poultry samples that have been irradiated. Chicken and turkey breast samples irradiated with cesium 137 at equal or greater than 1 kGy at 5°C can be detected by analyzing for a carbonyl

generated by the process. Within a few hours after irradiation it is possible to detect irradiated samples with a high degree of accuracy. Moreover, the dose the tissue had received can also be predicted. Upon storage of the irradiated tissue at -18°C for up to 5 weeks the samples that had been irradiated at 6 to 10 kGy lost more than 50% of that carbonyl content, but samples receiving doses of 1 to 3 kGy lost little, if any. The amounts of this carbonyl are considered to be below toxic levels. Thus, it is still possible to differentiate irradiated samples from controls. A single analyst can screen 12 samples in about 1 1/2 hours. Modification of the carbonyl test to make it suitable for detection of irradiated red meats will be investigated.

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SURFACE PASTEURIZATION

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CRIS #: 1935-41000-015
FSIS #:
Completion Date March 19, 1996

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215-233-6589

OBJECTIVE:

To develop a rapid and economic method for reducing the level of microbial contamination on the surface of poultry meat without introducing significant degradation of the quality of the product.

PROGRESS:

A device has been conceived, designed, and built to test the feasibility of treating raw poultry meat with steam so briefly that surface organisms are killed; but no appreciable cooking of the meat occurs. This is done by removing the air intervening between the steam and the meat; then cooling the meat by evaporating the steam condensate without air interference. The device is a chamber which can be rotated quickly from air, to vacuum, to steam, to vacuum, and back to air. It has proven capable of surface temperature rise as fast as 50,000°F (27,000°C) per minute; and comparable temperature fall rate. The tests using chicken meat, with and without skin, sterilized, then painted with *Listeria innocua* will begin as soon as sample holders are installed. A practical device, which will include provision for wrapping the treated piece, has been designed. This design is intended for transfer to a user.

PUBLICATIONS:

Morgan, A.I. 1994. Method and Apparatus for Treating and Packaging Raw (Poultry) Meat. US Patent 5,281,428. 20 claims. Issued Jan 25, 1994. Assigned to USDA.

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FOODBORNE VIRUSES

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FSIS #: I-90-2
Completion Date: February 6, 1996

OBJECTIVE A:

Develop sensitive methods to detect and differentiate virulent and inactivated viral human pathogens in meat, poultry, and by-products following gamma and electron irradiation using rapid nucleic acid-based tests.

PROGRESS A:

Work continues to evaluate the effect of gamma and electron irradiation on the nucleic acids of viruses which are pathogenic to people eating contaminated foodstuffs. The purpose of this study is to examine the effects of radiation to determine how inactivation takes place and to develop sensitive and accurate methods to differentiate between nucleic acids from virulent and radiation inactivated virus. A method to differentiate between active and inactivated viral nucleic acids is important because the very sensitive current nucleic acid-based methods used to detect viruses may not work effectively on irradiated products. A nucleic acid-based detection method is needed because some of the pathogenic viruses will not grow in cell cultures (Norwalk virus) and others are potentially hazardous for laboratory and regulatory personnel (Hepatitis A and B). These studies are being conducted with animal viruses which are biochemically and morphologically similar to the human viruses but are safe for humans. These include feline calicicvirus (FCV) which is a model for Norwalk virus, Hepatitis A, and other enteroviruses. Bovine viral diarrhea virus (BVDV) is being used as a model for Hepatitis B virus and other enveloped viruses. By using these viruses, methods development will be expedited. Studies with FCV and BVDV have shown that irradiation at very high levels (5 to 10 kGy) kills between 99.9 and 99.99% of these viruses; however, there is always a small number of surviving particles. We have been unable to inactivate all virus in our experimental samples. For characterization of the inactivation of the virus particles, we have developed sensitive PCR assays to examine the effects of irradiation on the viral genomes. This work has demonstrated that amount of amplifiable template decreases with increasing radiation dose. This decrease in template is more pronounced when the size of the area being amplified is larger. The decrease in product is greatest with large regions of 500 bases or more. This effect may be useful for differentiation between radiation inactivated and virulent-live virus.

OBJECTIVE B:

Develop oligonucleotide primers for use in nucleic acid amplification test that will detect Norwalk and Norwalk-like viruses in or on meat products.

PROGRESS B:

The major foodborne viral pathogen, Norwalk virus, belongs to the Calicivirus family. It causes severe gastroenteritis characterized by vomiting and diarrhea. Work on Norwalk virus has been hampered by the inability to grow it in cell culture. For research purposes, Norwalk virus is obtained by infecting human volunteers with known positive fecal samples. This makes it very difficult to work directly with Norwalk virus. To characterize this virus in greater detail, a model system is required which mimics its replication and growth. One virus for a possible model is the porcine enteric calicivirus (PEC) which causes gastroenteritis in pigs. It can be propagated in culture, although rather poorly, and will serve as an *in vitro* model for Norwalk virus. Because PEC causes a disease in pigs which is very similar to that of Norwalk virus in humans, there is some concern that this virus could be spread in meat products and cause human disease. Collaborations are being initiated to obtain PEC virus from clinical samples and to begin work examining this model system in greater detail. We already have started to develop reagents which can be used to amplify specific regions of the genomic RNA of Norwalk and related viruses. These reagents might provide a very sensitive means to detect this group of viruses in food products as well to detect them in environmental samples once foodborne disease is confirmed. If these methods prove successful, similar procedures will be developed for other human viral pathogens. The envisioned technology would require only limited space and expertise to perform, but may require one day to complete testing. Similar technologies have been shown useful for detecting human viral pathogens in well water and swimming pools.

PUBLICATIONS:

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ADVANCED TECHNOLOGIES FOR RESIDUE DETECTION

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CRIS #: 1935-42000-019
FSIS #: I-2
Completion date: April 15, 1996

OBJECTIVE A:

Develop Supercritical Fluid Extractor instrument suitable for use in regulatory laboratories.

PROGRESS A:

A prototype of the Applied Separations, Inc. Supercritical Fluid Extractor (SFE) was assembled in this laboratory as part of a CRADA agreement between ARS and the company. The prototype SFE was subjected to an extensive study to determine the instrumental specifications for the production model. The prototype performed well in all tests conducted with tissue samples and should meet the requirements needed in surveillance laboratories. The SFE is now in full commercial production. Agencies such as ARS, FSIS, and FDA have purchased these units for placement in their laboratories. The use of a common instrument in these laboratories should facilitate the development of advanced methodology of benefit to all agencies. Additional components needed to increase the performance of the SFE are jointly under development between ARS and the company.

OBJECTIVE B:

Apply SFE for the isolation of veterinary drug residues in animal tissues and fluids.

PROGRESS B:

A cooperative study was completed with a visiting scientist from the RIVM/ARO laboratory in the Netherlands. The focus of the study was to recover anabolic steroids from animal tissues and bovine urine by SFE. In multiresidue experiments nortestosterone, testosterone and methyltestosterone were added to liver at various fortification levels and extracted using supercritical carbon dioxide. The analytes were trapped on a sorbent bed (in-line) contained in the same vessel as the sample matrix. This technique traps the analytes of interest while most of the unwanted extracted material passes through this sorbent bed and is trapped downstream after CO_2 decompression (off-line). In initial studies the analytes were fortified in liver at a high level (500 ppm). The technique was ultimately refined for bovine urine so that the final fortification level was 12 ppm while recoveries for the three steroids were above 90% for each. A collaborative investigation between this laboratory and the RIVM is anticipated that will complete the investigation of the SFE of steroids in tissue samples.

Improved recoveries and detectability of three sulfonamides from chicken tissue by SFE, without modifiers, using an in-line adsorption trap has been achieved. Following SFE, the analytes are recovered from the neutral Alumina trap with the HPLC mobile phase. Samples are injected directly onto the HPLC column without post-extraction clean-up. Mean recoveries of sulfamethazine, sulfadimethoxine and sulfaquinoxaline from liver, breast tissue and thigh muscle are 89, 95 and 77% respectively. The analytes are detectable at less than 100 ppb with a minimum of background interference. Studies which included the more polar sulfathiazole in the extraction procedure have been initiated.

The role of water in the SFE of Zoalene and it's metabolites from chicken liver has been established. Dehydration of the tissue matrix during SFE results in adsorption of the analytes which limit their extraction. Adding 0.5 mL of water to the sample matrix prior to SFE prolonged the effective extraction period, thereby increasing the recoveries of Zoalene and three of its metabolites derivative from liver-Na_2SO_4 mixture with added water at 60° C, 15,000 psi and 3 L/min. CO_2 (expanded gas) for 30 minutes were 87, 92, 77 and 66% respectively. SFE can readily be employed by regulatory agencies as a substitute for solvent extraction of these residues from tissue.

Preliminary studies have demonstrated the feasibility of using SFE with in-line adsorption for extracting and recovering melengesterol acetate from tissue. Successful application of SFE to the analyses of this hormone at the ppb level would replace the current procedure which utilizes greater than 2 liters of organic solvent per sample. Studies are planned to extract this compound from tissue and fat samples by SFE at the tolerance level of 25 ppb.

OBJECTIVE C:

Develop alternatives to organic solvent extraction of extraction of sulfonamides from tissues.

PROGRESS C:

A procedure for the aqueous extraction of four sulfonamides from edible tissues and their detection at the 10-20 ppb level has been developed. Tissues are suspended in pH 8 buffer and "cooked" in a 100°C water bath to precipitate the proteins. Following centrifugation, the recovered supernatant is passed through an ion-exchange column buffered at pH 8. The trapped sulfonamides are eluted from the column with 0.5 N HCl-acetone which is diluted with water and passed through a neutral alumina column. Sulfonamide residues are reacted with fluorescamine and the fluorescent derivatives separated on a 15 cm C18 HPLC column. Detection is accomplished at an excitation wavelength of 366 nm and emission wavelength of 485 nm. Recoveries are greater than 90% for sulfathiazole, sulfamethazine and sulfadiazine and 70% for sulfaquinoxaline. Further experiments are planned to acquire replicate data on the isolation of these sulfonamides at the 10-20 ppb level.

OBJECTIVE D:

To collaborate directly with FSIS laboratories.

PROGRESS D:

In an expansion of the scientist exchange program between the two agencies begun last year, four ERRC scientist visited the FSIS Midwestern laboratory, St. Louis, MO to learn the priority needs of the scientists at that facility and to begin the process of technology transfer. During the visit, it was determined several high priority analytes determined by conventional methodology at that facility may be applicable to technology developed at ERRC. Four scientists from the FSIS Midwestern laboratory then visited ERRC in September to gain further insight into our research program and to continue discussions begun at their facility. As a result of this exchange program, collaborative research efforts in several areas are now underway. Additionally, a scientist from the FSIS Western laboratory visited ERRC for two weeks in late September for an in-depth training program in capillary electrophoresis and supercritical fluid extraction for eventual implementation of these technologies in that laboratory. Subsequently, an ERRC chemist visited the St. Louis FSIS Midwestern laboratory to demonstrate the use of the Applied Separations, Inc. SFE for trace residue recoveries. During that visit, the Chemist also demonstrated the immunoaffinity columns for β-agonists, which were obtained from a visiting scientist from the RIVM in the Netherlands. An ERRC scientist also visited the FSIS facility in Athens, GA. Discussions with microbiologists at that laboratory indicated a mutual interest in developing an enzyme modification technique to detect unidentified microbial inhibitors (UMI's). Collaborative studies between scientists at the two agencies will continue. Visits to other FSIS facilities are planned.

OBJECTIVE E:

Develop rapid screening tests for chemical residues.

PROGRESS E:

The specificity of the rapid herbicide screening assay was studied using metabolites and degradation products of atrazine (ATZ) as potential interfering compounds. Five compounds, 2-hydroxy ATZ, desethyl-2-hydroxy ATZ, desethyl ATZ, desisopropyl ATZ, and desethyl-desisopropyl ATZ, were examined at concentrations ranging from 5 ng/mL to 1000 ng/mL. Only desethyl ATZ gave a significant response in this concentration range, and then only at concentrations above 200 ng/mL. These results indicate that while the assay is capable of detecting a wide range of photosystem II inhibiting herbicides, it is also remarkably free from interference from close structural analogs such as metabolites and degradation products. No further studies in this area are planned.

A rapid test for the ionophore antibiotics salinomycin and lasalocid at a detection limit of less than 40 ng/mL (in buffer) was developed. The test uses lecithin: stearylamine: cholesterol liposomes containing terbium chloride at 10 mM as a reagent. The test is conducted by mixing the sample with liposome suspension and picolinic acid solution, and monitoring the phosphorescence at 590 nm. Ionophores in the sample mediated transport of terbium out of the liposomes and into solution, where the terbium was complexed by picolinic acid and formed a phosphorescent complex. Measurement of the phosphorescence at a fixed time (5-20 min) after mixing provided a measure of the ionophore concentration. Future work will investigate performance of this test with spiked biological samples.

OBJECTIVE F:

Detection of pathogenic bacteria in meat using immunoelectrochemical sensors.

PROGRESS F:

Research was initiated on rapid methods for detection of pathogenic bacteria in meat using immunoelectrochemical sensors. ELISA methods for evaluation of avidity and cross reactivity of antibodies have been developed, and sensor electrodes containing the immobilized antibodies have been prepared and characterized. Planned research will focus on evaluating specificity of the technique for individual serovars such as *E. coli* O157:H7 in the presence of other *E. coli* types.

In a related investigation, a dual crystal flow-through cell has been designed and built and will be used with piezoelectric crystals, coated with antibodies to bacterial or antibodies to toxic cellular products. In planned experiments, a dual quartz crystal microbalance oscillator circuit is being constructed and will be used with this detector. This circuit will provide accurate measurements of frequency differences between the two crystals, each with one side immersed in the liquid of the flow-through cell and will compensate for the effects of temperature and conductivity of the medium in the cell.

OBJECTIVE G:

Detection of antibiotics using non-immunochemical methods.

PROGRESS G:

Fluorescent non-immuno competitive protein binding assay is being developed for the quantitation of penicillins. The binding protein is labeled with FITC fluorescein isothiocyanate and the competing analyte is covalently bound to polystyrene particles. The penicillin analyte in the standard or sample is allowed to compete with the bound penicillin for the binding sites of the protein. The excess reagents are separated by vacuum filtration and the fluorescent signals from the FITC-binding protein-penicillin-particle complex are determined. This assay showed detectability of penicillin G at 0-50 ppb in buffers and

1.25-25 ppb in kidney extract with added penicillin G. This assay will be applied to liver and kidney samples containing incurred penicillins. This technique can quantitatively analyze 25- 40 samples in less than 1 hour. There is also an effort in ERRC to reduce the number of UMI (unidentified microbial inhibitors) compounds by developing identification methods. Utilizing an enzyme modification technique, cephalosporin compounds can be identified. Collaboration with FSIS laboratories is being sought for the utilization of this technique in the regional laboratories (ERL and MWL).

Studies leading to the development of a non-immunoaffinity method using immobilized receptors for multiresidue isolation of β-lactam antibiotics from animal tissues and fluids continues. An affinity gel matrix containing a protein (P-BA3) described in earlier reports remains the center of focus for these studies. Earlier work demonstrated the binding efficiencies and selectivity of P-BA3 for various β-lactam drugs, examined the possibility of side product formation, and established the stability and regeneration of the gel for multiple use. In current studies, a more detailed examination of the physical nature of the binding interaction between the drug and immobilized protein is made. It has been determined that the mode of immobilization (i.e., type of immobilization support), pH and ionic strength play essential roles in influencing the protein's ability to bind the drug. Temperature (25°-37°C) appears not to have an effect on drug binding. In a novel approach, the thermal energetics of the binding reaction and the stability of the protein affinity matrix were studied by microcalorimetry. These types of studies have given a better understanding of the receptor-drug interaction at the basic level and should aid in predicting the performance of a given biomolecule as a receptor for β-lactam antibiotics in either the soluble or immobilized form. Based on these results, new strategies for immobilizing P-BA3 are being tested which would hopefully enhance its binding to antibiotics. Four other proteins have been evaluated in solution for their effectiveness as β-lactam affinity receptors. Three of these proteins may possibly act as low-affinity receptors for benzylpenicillin, ampicillin and hetacillin. Continuing efforts will be made to elucidate their drug-binding behavior in both the soluble and immobilized forms.

OBJECTIVE H:

Develop rapid screening methods for the detection of spectinomycin and hygromycin B.

PROGRESS H:

A polyclonal antibody against hygromycin B was produced with titers of 1/1000 - 1/2000. This antibody titer is better than the antibody purchased commercially which had a low titer (less than 1/1000) and low affinity. Approximately 950 mL of antiserum is in storage which is available for 28 million tests. In future experiments, this antibody will be characterized and its use will be optimized for development of a screening assay and affinity clean-up of hygromycin B. The antisera production for spectinomycin was not successful. However, a novel assay approach is under development for the detection of spectinomycin utilizing a binding protein. This protein was previously utilized at ERRC for the affinity capture of

aminoglycosides and was reported in the 1993 update report. The binding protein binds spectinomycin in a concentration 1000 times less than its binding for hygromycin. The assay utilizes a particle concentration competitive binding protein technique. Fluorescent labeling and purification of the labeled spectinomycin; the covalent attachment of the binding protein to the polystyrene particles and assay conditions are being optimized. Spectinomycin was detected from 0 - 50 ppb in buffers and preliminary research showed detectability of 2 -25 ppb spectinomycin fortified in plasma and kidney extract. In planned experiments, the sample preparation technique will be modified and optimized in order to avoid clogging of filter membranes. This method potentially can analyze 25-40 samples/hour.

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DEVELOP IMMUNOCHEMICAL-BASED RESIDUE METHODS FOR ANALYSIS OF VETERINARY DRUG AND PESTICIDE RESIDUES IN FOOD ANIMAL PRODUCTS

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CRIS #: 6202-42000-005
FSIS #: I-2
Completion date March 4, 1994

OBJECTIVE A:

Develop, evaluate, and provide confirmatory testing of monoclonal antibody-based enzyme linked immunosorbent assays (ELISAs) that will be useful for on-the-farm or in-the-processing plant analysis of pesticide/drug residues in animal products and body fluids.

PROGRESS A:

Development of monoclonal antibody based immunoassays are in progress for the following compounds: halofuginone, hygromycin B, pirlimycin, fumonisin, furosemide, beta-lactam antibiotics (ceftiofur), salinomycin, monensin, sulfa drugs (e.g., sulfamethazine), dioxin, and carbadox. Many of these have been identified by FSIS as being important for development of immunoassays. The immunoassays for halofuginone, salinomycin, and hygromycin B are the most complete.

Halofuginone: The halofuginone immunoassay is formatted as an ELISA with sensitivities in the 1 to 10 ppb range. Analysis of fortified chicken serum samples has been completed with 90-100% recoveries. Incurred chicken liver and serum samples have been generated. These are being evaluated using the ELISA and an HPLC method developed in our laboratory.

Salinomycin: The salinomycin immunoassay is also formatted as an ELISA. Sensitivities are in the low ppb range. This assay is being targeted for chicken liver. In its present format, the assay does not require any complex sample preparation or depend on the use of organic solvents. The liver sample is simply homogenized in buffer, diluted, and assayed. Studies using fortified liver samples resulted in recoveries of 80-100%. Incurred liver samples are currently being tested. The ELISA results from the spiked liver studies have been compared to results obtained on split samples using an HPLC method adapted for chicken liver. Similar comparisons will be made for the incurred liver samples. A US patent for the salinomycin ELISA has been applied for and the ELISA as been exclusively licensed to Neogen Inc. They anticipate marketing immunoassay kits by the end of this calendar year.

Hygromycin B: An ELISA has been developed for Hygromycin B, an approved feed additive used to control worm infestations in swine and poultry. Studies using fortified swine kidney samples (in the 1-4 ppm range) have been completed. Using a simple aqueous extraction procedure recoveries of 80-91% were observed. A patent application is being prepared for these monoclonal antibodies.

Other Compounds: Monoclonal antibodies have been developed for monensin. They are currently being evaluated by one of our CRADA partners. We have developed an immunoassay for furosemide. It is currently being evaluated for its ability to detect furosemide residues in milk. An immunoassay for fumonisin has been developed with detection limits in the low ppb range. The assay detects fumonisin B1, B2 and B3. We have completed a number of cell fusion experiments in an effort to isolate monoclonal antibodies for pirlimycin and ceftiofur. Antibodies with sufficient affinities have not yet been identified. New immunogens are being supplied by one of our CRADA partners in an effort to generate the needed antibodies. We have generated a new immunogen in an effort to produce monoclonal antibodies to the sulfa drugs; e.g., sulfamethazine. This conjugate has been synthesized de novo and should result in antibodies capable of recognizing most members of this important class of animal drugs. The immunoassay for the sulfa drugs is being developed in collaboration with a CRADA partner. We also have synthesized a new immunogen for carbadox. As with the sulfa drugs, the carbadox immunogen represents a *de novo* synthetic effort and this synthesis was necessary in order to optimally position the site for linkage to carrier protein. Finally, we are collaborating with Dr. Bruce Hammock, University of California-Davis in an effort to produce anti-dioxin monoclonal antibodies. We have generated a group of immunized animals using conjugates supplied by Dr. Hammock.

OBJECTIVE B:

Develop classical analytical methodologies (e.g., HPLC, GLC) for the analysis of pesticide/drug residues to provide confirmatory testing of the immunoassays.

PROGRESS B:

Two HPLC methods have been developed to support our studies on the development of immunoassays. An extraction and HPLC method that is able to detect halofuginone in chicken serum has been developed. The method has a detection limit of 1.5 ng/mL, and recoveries of 96-99% were observed in samples spiked at 10 ng/mL. Chickens fed halofuginone at 3 ppm for 10 days resulted in 3.75 ng/mL Halofuginone in the serum. Secondly, an HPLC method for detection of salinomycin in grain has been adapted for detection of drug residue in chicken liver. The method utilizes post column derivitation with vanillin and has a detection limit of 50 to 100 ng/g.

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DEVELOP MULTIPLE RESIDUE IDENTIFICATION METHODS FOR TESTING FOOD ANIMAL TISSUES

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CRIS #: 1280-42000-009
FSIS #: I-2
Completion date January 1, 1997

OBJECTIVE A:

Develop simple and rapid physical-chemical procedures for detection and confirmation of antibiotic residues in animal products at levels of concern to regulatory agencies, and verify methods using incurred residues from treated animals.

PROGRESS A:

Work was begun on adapting the multiresidue procedure developed for determination of β-lactam antibiotic residues in milk to residues in tissues. A modified method was developed for extraction of residues from tissue homogenates using tetraethylammonium chloride and acetonitrile. The acetonitrile was removed by evaporation and the extract was concentrated on an HPLC column from water. The β-lactams were eluted with an acetonitrile gradient. Fractions containing each β-lactam were collected and analyzed. The HPLC fractions could be tested by screening tests used for milk testing and further analyzed by HPLC. The test kits worked with ampicillin, amoxicillin, penicillin G, cloxacillin, and cephapirin at levels of 10 ppb. Ampicillin and penicillin G could be determined by HPLC at 10 ppb. Cephapirin was converted to its desacetyl metabolite in tissue homogenates. It could also be determined by HPLC at levels of 10 ppb.

Work was completed on a rapid method for tetracycline in tissues. Tissues were extracted with 0.25 NHC1-acetonitrile. Hexane and methylene chloride were added to separate the water layer. The water layer was concentrated and injected for isocratic analysis on a Polymer Laboratories PLRP-S column. The HPLC mobile-phases used phosphoric acid with alkylsulfonates added as ion-pairs to separate tetracyclines from interferences. This eliminated the need for further sample cleanup. Recoveries ranged from 70-100 percent depending on the compound and substrate. The limits of quantitation were about 20 ppb for oxytetracycline and tetracycline and 50 ppb for chlortetracycline. The procedure was evaluated with beef and pork muscle, liver, and kidney.

PUBLICATIONS:

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DEVELOPMENT OF METHODS FOR RESIDUE ANALYSIS

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CRIS #: 1270-42000-002
FSIS #: I-2
Completion date: November 27,1994

OBJECTIVE:

Develop rapid, inexpensive, and uncomplicated analytical methods to screen for pesticides in meat at tolerance levels.

PROGRESS:

Determining Propoxur and other carbamates in meat using HPLC-fluorescence and gas chromatography/ion trap mass spectrometry after supercritical fluid extraction:

Supercritical fluid extraction (SFE) using CO_2 was examined for separating carbamate pesticides from interfering coextractives prior to analysis by either liquid chromatography with fluorescence detection or by gas chromatography/ion trap mass spectrometry(GC/ITMS). Preextraction of the ground meat with acetonitrile prior to SFE left behind over 99% of the fat and fiber in ground meat. The concentrated acetonitrile extracts containing the carbamates and other coextractives were adsorbed on a pelletized diatomaceous earth for SFE. SFE further reduced the amount of co-extractives 10-fold. This procedure allows larger, more representative samples to be routinely analyzed, removes interferences that appear in the fluorescence mode, and reduces non-volatiles that can accumulate on the capillary column in the GC-ITMS mode shortening its lifetime after multiple injections. The application of supercritical fluid extraction to residues in animal tissues has been limited by the small sample sizes that can be used and by coextraction of fat which must be removed prior to analysis. The present procedure, by using a preliminary acetonitrile extraction, overcomes both of these problems.

Supercritical Fluid Extraction (SFE) of fruits and vegetables:

A method of multiresidue analysis using SFE and GC/ion trap MS detection for 53 pesticides was developed and tested in fruits and vegetables. This procedure uses 2 g subsamples taken from homogenized 50 g representative samples and requires only 1.5 mL of acetonitrile to remove the extracted residue from the sorbent trap. No additional cleanup is required for fruits and vegetables. Samples with high lipid content require additional clean-up.

Flow Immunosensor:

The flow immunosensor works by immobilizing an antibody on a column of glass beads, incubating the antibody with an antigen-dye conjugate, and upon injection of true antigen in a flowing system, the antigen-dye is displaced and measured by fluorescence downstream. The device is potentially less tedious, less expensive, and more rapid than ELISA techniques. Results with alachlor, using monoclonal antibody produced by Dr. Sara F. Wright (USDA ARS, Beltsville), showed that it was possible to detect 10 ng/mL alachlor in the mobile phase buffer. However, the parent compound, alachlor, does not occur in meat, and the low cross-reactivity of the antibody for alachlor metabolites, including 2,6-diethylaniline, prevented rapid analysis of real samples at tolerance levels (20 ng/g in meat). Monsanto has developed an antibody with high cross-reactivity for alachlor metabolites, but FSIS (Athens, GA) findings have shown that alachlor does not occur in meat. A more practical application of the flow immunosensor is being developed in collaboration with Dr. Larry Stanker's group (USDA ARS, College Station, TX). A second, more compact prototype of the flow immunsensor was manufactured, more columns, commercial oxirane beads, and the succinimidyl mono-ester form of the Cy-5 dye were obtained for the research.

PUBLICATIONS:

Robert J. Argauer, Konstantin I. Eller, Medina A. Ibrahim, and Richard T. Brown. Determining Propoxur and other Carbamates in Meat Using HPLC-Fluorescence and Gas Chromatography/Ion Trap Mass Spectrometry after Supercritical Fluid Extraction. *J. Agric. Food Chem.* (Submitted).

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO RESIDUE DETECTION

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CRIS #: 3620-42000-013
FSIS #: I-2
Completion Date: December 17,1998

OBJECTIVE A:

Develop supercritical fluid-based techniques for sample cleanup prior to residue analysis.

PROGRESS A:

Research efforts this past year have focused on the development of off-line and on-line methods for sample cleanup as well as extraction. The primary focus of this research has been to minimize or eliminate interfering lipid and fat material which coextracts with the target analyte into the supercritical fluid. Size exclusion chromatography (SEC), utilizing CO_2 at high pressure as a partial replacement solvent for methylene chloride shows considerable promise. Several SEC columns were tested for compatibility with CO_2 or mixed CO_2/cosolvent eluents at high pressure (200-350 atm) during this reporting period. Separations, identical to those recorded with liquid eluents, could be achieved using 100 and 500 Angstrom Jordi Gel columns available from Jordi Associates. Fractionation of lipid material from pesticides and PAHs could not be achieved with pure CO_2; however, excellent separations were achieved with mixed phases containing over 80% SC-CO_2 in place of the methylene chloride.

Another SEC alternative that is being investigated is the replacement of methylene chloride with other organic eluents. Over ten alternative solvents have been screened with respect to producing similar elution patterns and fractionation of pesticide/fat moieties as achieved with 100% methylene chloride. Three solvents: tetrahydrofuran, ethyl acetate, and p-dioxane have proved satisfactory, based on the elution patterns on the Jordi Gel columns of lard and 4-5 pesticides of each of the following pesticide classes: carbamates, organochlorine, organophosphorus, and triazines.

Additional experiments have also been run on the new Hewlett Packard SFC to test the effect of SC-CO_2 substitution on the retention and separation of the above pesticide classes in tetrahydrofuran, ethyl acetate, p-dioxane, and methylene chloride. Using the above pesticide/lard mixtures, rather than the previously used EPA-based calibration standard, it was observed that certain intermediate compositions of SC-CO_2 with organic solvent substantially reduced the amount of organic solvent required for the separation. The physicochemical basis of this solvent reduction is related to the relative contributions of adsorption and size

exclusion to the retention of solutes on the hydrophobic Jordi Gel columns. Studies of resin expansion in the above organic eluents also indicate the importance of this phenomena for achieving the critical separation of pesticides and fat.

Another promising approach for minimizing lipid coextractives is the use of fluoroform, HCF3, in place of SC-CO_2. Research in our laboratory has shown that SC-HCF_3 extracts 100-fold less fat than SC-CO_2, while yielding similar recovery efficiencies of pesticides from meat matrices. This is a result of the lower solubility parameter of fluoroform relative to those values recorded for typical fat triglycerides. This research has been accomplished in conjunction with Air Products & Chemicals, Inc.

On-line cleanup of fat-containing supercritical fluid extracts (SFE) has been successfully accomplished on gram-sized meat samples, such as adipose tissue and liver, utilizing a SFE-SFC-GC system constructed from commercial instrumentation. Excellent results have been achieved to date using both EC and N-P detection for organo-chlorine and -phosphorus pesticides. Detection has been successfully achieved below the FSIS LDL values with excellent precision (RSD's below 5%) with both detectors. The technique uses modest extraction conditions to minimize the amount of coextracted fat, followed by extract cleanup on C8 microbore column with SC-CO_2 as the eluent.

OBJECTIVE B:

Integrate Supercritical fluid extracts with enzyme immunoassay for the rapid, environmentally acceptable assay of pesticides in meats.

PROGRESS B:

Studies have been completed on the coupling of SFE with immunoassay for the quantitative determination of pesticides in meat samples. During the course of this research, several pumpless extractors and fluid-filling schemes were developed at NCAUR for doing static SFE in the field. Dynamic extractions on the same meat samples have also been conducted on a Dionex Model 723 SFE unit. Magnetic bead-based ELISA kits (Ohmicron) for alachlor, carbofuran, atrazine, benomyl, and 2,4-D were used to determine the pesticide content of the supercritical fluid extracts. Pesticide recoveries were all above 90% at minimum detection limits below the FSIS-specified LDL values. A key step in developing these quantitative assays was the inclusion of a simple microfiltration step of the extracts before performing EIA. The manuscript describing this research has been published in the Journal of Agricultural & Food Chemistry. No further research is planned in this area at this time.

OBJECTIVE C:

Evaluation and development of supercritical fluid extraction equipment for use by FSIS.

PROGRESS C:

Evaluation of a new Hewlett Packard SFC has been on-going during this reporting period for pesticide and nutrient analysis. Studies on pesticides have largely focused on establishing the separation conditions and detection limits for selected carbamate pesticides. Using NPD detection, the SFC/NPD can detect 3-5 ng of carbofuran on-column with the present injection loop and column. This is still about ten-fold more than can be detected using our HPLC post column derivatization method. The unit has been recently outfitted with FID capability. Several new pieces of equipment were purchased for use in the program. These include a Varian GC/MS/MS ion trap system, an Applied Separations extractor, HP data station, HP gas chromatograph, HP 7680T extractor with cosolvent capability, a new DS for the Incos 50 MS, and a Sedex light scattering detector. We are currently beta-site testing the Isco 3560 multi-sample extractor and anticipate the arrival of a SuprexAutoprep 44 multi-sample extractor beta-site instrument in September of 1994. At the request of FSIS's Chemistry Division, assistance was provided to FSIS's Western Laboratory in Alameda in learning the operation of the multi-sample extractor installed over two years ago at the Western Laboratory. An instruction manual was written for the instrument and on-site training provided by James Johnson of our laboratory.

OBJECTIVE D:

Organization of meetings and activities of interest to FSIS.

PROGRESS D:

The 5th International Symposium on SFC and SFE was held in Baltimore, MD on June 10-14, 1994. J. King served as the program chairman and on the organizing committee for the symposium. NCAUR's supercritical fluid research team presented six papers; three oral and three posters. The symposium attracted approximately 260 attendees who heard approximately 47 oral presentations and saw over 120 posters. An instrument exhibit held in conjunction with the symposium featured the latest in SFE and SFC equipment. NCAUR's exhibit entitled "Supercritical Fluid Technology", stressing ARS's role in technology transfer, was well received by the many attendees who stopped at the booth, including Cindy Deyrup and Dave Soderberg from FSIS. The next stateside SFC/SFE symposium will be held in Indianapolis during the month of April, 1996.

A symposium entitled, "New Approaches to Sample Cleanup and Extraction" has been organized by J. King for the upcoming Pittcon Conference in New Orleans in 1996. Speakers include Jack Henion of Cornell, Willie Hinze of Wake Forest, Jeff Brewster of ERRC, Jacelyn Pare of Environment Canada, Janusz Pawliszyn of the University of Waterloo, and Steven Barker of LSU. Topics that will be covered include surfactant media phase separations, fibre sampling, on-line immunoaffinity chromatography with MS, and microwave assisted extraction processes. A symposium on analytical uses of SFE and SFC is also being organized for the 1995 American Oil Chemists Society Meeting to be held in

San Antonio, Texas by Janet Snyder. Several of the invited speakers are from ARS and will be speaking on subjects of interest to FSIS, such as SFE of toxicants, nutritional labeling analysis, and the analysis of volatiles/semivolatiles. An AOAC associate referee's report on the SFE of meat products was filed with general referee, Dave Soderberg, during this past reporting period. This CRIS, which addresses FSIS needs in supercritical fluid technology, terminated in January, 1994. However a new CRIS proposal has been written, reviewed by professional peers, and was authorized by ARS-NPS for continued funding.

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IMMUNOCHEMICAL METHODS FOR PESTICIDES

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CRIS Number: 5325-42520-001
FSIS Number: 1-2
Completion Date: January 1997

OBJECTIVE:

Apply immunochemical and biosensor methods to quantify components of foods which influence the safety and healthfulness of the food supply.

PROGRESS:

This subproject builds on the previous work done to develop screening assays for benzimidazole antihelmintics, including thiabendazole, fenbendazole, oxfenbendazole, oxibendazole, cambendazole, and albendazole. Monoclonal antibodies were developed for both major groups of compounds, the thiazolyl benzimidazoles (thiabendazole, its 5-OH metabolite, and cambendazole) and the methyl benzimidazolecarbamates (including albendazole, fenbendazole, oxfendazole, and the benomyl breakdown product, carbendazim). ELISA's were developed and used to analyze bovine liver samples and produce matrices. Simple aqueous extraction protocols were developed for the parent compound and metabolites in liver and for the fungicide residues in produce. Use of 80% methanol for extraction of thiabendazole from produce samples had some advantages in reducing matrix effects and permitting a more rapid assay format to be used. Detection limits using these techniques ranged from 10-130 ppb, depending on analyte and matrix. The assays have sufficient sensitivity and appropriate specificity to serve as screening tools for field or laboratory use. An antibody immobilization protocol was developed and tested with three different silicon-based substrates. A stable antibody density of 2×10^{11} molecules per cm^2 was demonstrated in this first phase of development of a homogeneous sensor system for direct detection and quantification of residues. Under a Cooperative Agreement recently initialed by both parties, Millipore Corporation (Bedford, MA) plans to market our thiabendazole method as part of the Envirogard immunoassay kit product line.

PUBLICATIONS:

Brandon, D. L., Binder, R. G., Bates, A. H. and Montague, W. C., Jr. 1994. Hapten strategy for thiabendazole and other benzimidazoles. In Kurtz, D. A., Skerritt, J. H., and Stanker, L. (Eds.) Agrochemical Immunoanalysis. AOAC Intl., Arlington, VA. (Accepted for publication).

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DOSED TISSUES AND FLUIDS (INCURRED RESIDUES) OF HERBICIDES, INSECTICIDES, AND OTHER CHEMICALS THAT MAY CONTAMINATE MEAT AND POULTRY

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CRIS #: 6202-42000-004
FSIS #: I-2
Completion date March 4, 1995

OBJECTIVE A:

To produce food-animal tissues and body fluids containing specified levels of incurred residues of veterinary drugs, pesticides, or other chemicals for use by FSIS in the development and validation of analytical methods.

PROGRESS A:

In CY-94, personnel at the Food Animal Protection Research Laboratory began a residue study using specific tranquilizers to produce incurred tissues for the purpose of refining and validating the performance of a method for the detection of the following tranquilizers: 1) Acepromazine, 2) Azaperone, 3) Chlorpromazine, and 4) Xylazine. Three market hogs (plus or minus 220 lbs) were injected intramuscularly (IM) with 0.22 mg/kg of Acepromazine and slaughtered within 20-30 minutes post-injection. Liver, kidney, and muscle samples were harvested, frozen, and sent to Carolyn Henry at the FSIS Midwestern Laboratory in St. Louis, MO for residue detection. Likewise, three market hogs were injected IM with 4 mg/kg of Chlorpromazine and tissues were collected 20-30 minutes post-injection, frozen, and submitted to the FSIS Midwestern Laboratory for residue detection. For the remainder of the study, three additional market hogs will be injected IM with 8 mg/kg of Azaperone and three calves will be injected IM with 0.35 mg/kg Xylazine. Tissues (liver, kidney, and muscle) will be harvested 30 minutes post injection on the hogs and 15-20 minutes post-injection on the calves and sent to the FSIS Midwestern Laboratory for residue detection.

PUBLICATIONS:

None

DISPOSITION OF DRUGS AND DIOXINS IN FARM ANIMALS.

ARS Contact Persons:
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CRIS #: 5442-32000-006
FSIS #: I-90-6
Completion date: February 1997

OBJECTIVE:

To develop more complete information on the disposition of drugs and dioxins in farm animals.

PROGRESS:

Sulfadimethoxine in Swine: The experimental design was as follows, eight, 25 to 40 kg barrows were dosed with 14C-sulfadimethoxine (60 mg/kg, orally, specific activity approximately 1000 dpm/fg) and two animals each were sacrificed at 12, 24, 48, and 72 hours after dosing. Tissues were collected, ground and the disposition of total radioactivity was determined by sample oxidation. Urine and feces were collected at 6 and 12 hours post dosing and then every 12 hours until sacrifice. Details of the tissue disposition and elimination of 14C-activity have been previously reported (see previous reports for details). More recent studies have demonstrated that the parent compound sulfadimethoxine (SDM) accounted for approximately 10% of the 14C-activity in the urine (the % varied somewhat depending upon the time interval after dosing). N4-Acetylsulfadimethoxine (SDM-Ac) was the major 14C-labeled fraction in the urine. Studies are currently underway (mass spectrometry, 1H-NMR and comparison with reference compounds) to identify two very polar metabolites and one or more nonpolar 14C-labeled metabolites isolated from the urine. Other studies currently underway indicate (based on chromatographic behavior) that SDM is the major 14C-labeled fraction in skeletal muscle along with smaller amounts of SDM-Ac, two more polar metabolites, and trace amounts of at least one nonpolar metabolite. Similar studies indicated that there was approximately equal amounts SDM, SDM-Ac, one very polar metabolite and one or more nonpolar metabolites in the liver. Studies are currently underway to conclusively identify (spectral methods) the 14C-labeled compounds isolated from the liver and skeletal muscle from swine dosed with 14C-sulfadimethoxine.

Levamisole in cattle: Studies to determine the disposition of the antihelmintic levamisole [1-2, 3, 5, 6 tetrahydro-6-phenylimidazo (2, 1-b) thiazole] in lactating dairy cows have been continued. One cow was orally dosed with 14C-levamisole and one cow was subcutaneously dosed with 14C-levamisole. Milk, blood, urine and feces samples were collected from 0-48 hours after dosing and tissues were collected when the animals were sacrificed 48 hours after dosing. Most of the 14C-activity was excreted in the urine (major route of excretion) and feces within 48 hours. The major 14C-labeled metabolite in the urine was isolated and identified as an oxidation product of 14C-levamisole (ring cleaved and the number two

carbon oxidized to carboxyl moiety). Four 14C-labeled compounds (parent compound and three metabolites) in milk have been isolated and identified by interpretation of MS and 1H-NMR spectra and comparison with reference compounds when available. Studies to characterize the 14C-labeled residues in liver have been initiated. Results to date indicate that levamisole accounted for only a small percentage of the 14C-activity in the livers from cows dosed both orally and subcutaneously with 14C-levamisole.

Ractopamine in turkeys and rats: Studies on the disposition of ractopamine in turkeys and rats have been concluded. Colostomized turkey poults were dosed with 14C-ractopamine (racemic mixture) and urine, feces, and tissues were collected after dosing. The turkeys excreted 52% of the 14C-activity in the urine and 42% of the 14C-activity in the feces within 48 hours after dosing and the carcass contained 3% of the dose 48 hours after dosing. The urine from the turkeys contained at least 12 metabolites, 6 of which were isolated and identified as regio-and stereo-isomeric glucuronic acid conjugates. The RS and RR isomers were preferentially conjugated with glucuronic acid (relative to conjugation of SR and SS isomers). Studies were also conducted to compare the biological activity of ractopamine when given orally and IP. Ractopamine, when given IP, increased average daily gain, net weight gain, and feed utilization in rats; however, ractopamine, when given orally, had no significant affect on these parameters. Subsequent studies demonstrated that 14C-activity given orally to rats as 14C-ractopamine was rapidly absorbed from the gastrointestinal tract (GIT). Thus the lack of biological activity of orally administered ractopamine was not due to poor absorption from the GIT. More recent studies involving isolation and identification of 14C-metabolites in the excreta and bile from rats dosed with 14C-ractopamine and enterohepatic circulation studies suggested that ractopamine was rapidly conjugated with glucuronic acid in the GIT (i.e. before entering general vascular system).

Clenbuterol in cattle: Studies to investigate the disposition of 14C-clenbuterol in the young ruminant animal have been planned. The synthesis of 14C-clenbuterol is currently underway (contract with Huntington Laboratory, United Kingdom). The metabolism studies will be initiated when the 14C-clenbuterol synthesis has been completed (estimate September 1994).

Dioxin in cattle and rats: We conducted a metabolic study on 1,2,7,8-tetrachlorodibenzo-p-dioxin (1278-TCDD) given orally (350 fg dioxin; 8.5 fCi [UL 7,8 ring 14C] per rat; about 54,000 dpm/fg) to four male Sprague-Dawley rats (weight 367-388 g). Seventy seven percent of the 14C was recovered in the feces, most of which was recovered in the first two days and 17% was recovered in the urine (11.7% in 0-24 hr). About 0.4% of the dose remained in the GIT. One percent of the dose remained in the kidneys, liver, and carcass combined. Residues in the brain, muscle, and perirenal fat were 56, 80 and 5118 dpm/g, respectively. Three urinary metabolites have been characterized. The first is a glucuronide of 4,5-dichlorocatechol representing about 19% of the urinary 14C. The second and third metabolites were 4,5-dichlorocatechol and monohydroxy-trichlorodibenzo-p-dioxin representing 42.3% and 3% of the urinary radioactivity, respectively. NMR studies are in progress to determine the location of the hydroxylation. We plan to conduct similar metabolism studies on the 1,3,7,8-, 1,4,7,8- and

2,3,7,8-TCDD and 1,2,3,7,8-pentaCDD. With regard to the study on dioxin levels in beef samples collected throughout the United States, we have in storage samples of cattle tissues from North Dakota, Nebraska, Montana and Oregon. These 105 tissue samples are from 21 cows with additional samples known to have been collected and in storage in Oregon, Indiana and Florida. Presently, we have 29 samples prepared for GC-MS analysis consisting of controls and appropriate reference standards. A dioxin feeding study is being planned for this fall to study the distribution (partitioning of various environmental dioxin congeners) in beef. Beef calves thought to be free of dioxins will be located and fed a concentrate supplement laced with known amounts of dioxins. The amounts of dioxins will be kept low, but near the amount expected to presently be occurring in some situations from flyash. The remainder of the diet for the calves will comprise concentrate and hay obtained and processed in a manner to keep environmental contamination with dioxins low. The calves will be slaughtered at the end of the study and various tissues will be harvested and assayed for the dioxin congeners.

Sulfathiazole in Swine: The experimental design was as follows, eight, 32-39 kg barrows were dosed with 14C-sulfathiazole (100 mg/kg, orally, specific activity approximately 3500 dpm/fg) and two animals each were sacrificed at 6, 12, 24 and 48 hours after dosing. Tissues were collected, ground and disposition of radioactivity determined by sample oxidation. Urine and feces were collected at 6 and 12 hours post dosing and then every 12 hours until sacrifice. Sulfathiazole (STZ), N4-acetylated-(NAC), N4-glucuronide- (NGRD) and N4-glucoside- (NGSD) metabolites have been isolated (solid phase extraction and HPLC) and identified (FAB mass spectrometry and 1H-NMR with comparison to known standards in the tissues). Levels of STZ, NAC, NGRD and NGSD were 16%, 22%, 7% and 38%, respectively, of the radioactivity in the liver 6-12 hours after dosing. Levels of STZ, NAC, NGRD and NGSD were 17%, 16%, 1.5% and 57%, respectively, of the radioactivity in muscle in 6-12 hours after dosing. Levels of STZ, NAC, NGRD and NGSD were 32%, 40%, 9%, and 3%, respectively, of the radioactivity present in the kidney 6-12 hours after dosing. Levels of radioactivity in these tissues 12 hours after dosing were too low for metabolite determination. Levels of STZ, NAC, NGRD and NGSD were 64%, 27%, 2% and 3%, respectively of the radioactivity in the 0-6 hr urine. Generally, STZ levels dropped and NAC levels increased in urine samples collected at later times. The 36-48 hr urine levels of STZ, NAC, HGRD and NGSD were 26%, 61%, 0.6% and 3.2%, respectively. STZ levels in skeletal muscle, heart, spleen, visceral fat and body fat were below 0.2 ppm after 48 hours. STZ levels in blood, lungs, gall bladder, and carcass were below 0.3 ppm after 48 hours. STZ levels in liver and kidneys were 1 and 1.9 ppm after 48 hours. Most of the 14C-STZ was excreted in the urine (84%) with a small amount excreted in the feces (3.5%) after 48 hours. A manuscript on this work has recently been written by Dr. P.W. Aschbacher and will be submitted for publication in the near future.

PUBLICATIONS:

Smith, D.J., J.M. Giddings, V.J. Feil and G.D. Paulson. Identification of ractopamine hydrochloride metabolites excreted in rat bile. Xenobiotica (To be submitted).

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PHARMACOKINETIC MODELS

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CRIS #: 1280-32000-001
FSIS #: I-89-1
Completion date: September 30, 1997

OBJECTIVE A:

To develop mathematical models of the pharmacokinetics of TCDD and related compounds in beef and dairy animals.

PROGRESS A:

The emphasis of this project was changed during the past year to address concerns about the background levels TCDD (2,3,7,8-tetrachloro-p- dibenzodioxin) and related compounds raised by the recent Environmental Protection Agency reassessment of dioxin-like compounds. The dioxin- like compounds are lipophilic, and depending on the number and positions of chlorine substitution, resistant to metabolic degradation. Literature on the behavior of dioxins in cattle is scant and often lacks basic information on feed intake, weight changes, levels of milk production, and fat content of milk. The data suggest, however, that many concepts developed from data on other halogenated hydrocarbons can be adapted to dioxins and related compounds.

Several published growth and feed intake models were examined to determine their suitability for developing a model for growing cattle similar to the model for growing pigs described in previous reports. The growth phase of the beef production cycle before animals enter feed lots is thought to be the most critical time for animal exposure to dioxin residues. The preliminary evaluation of the growth models indicate that the beef models are less satisfactory than the models available for pigs and that modifications will be required. Another area of difficulty is the necessity to properly evaluate the significance of the growth rates that occur when the amount of pasture or other feed is restricted. A cooperative agreement is being established with the Ohio Agricultural Research and Development Center to enhance the rate of progress on this project.

Residue simulations using the pig model indicated that changing genotype of the animals or energy density of the diets in order to produce leaner animals could have significant effects on the residue concentrations in the carcass. A set of samples from pigs administered porcine growth hormone, which produced a marked change in body composition with no change in feed intake, are available from a Beltsville experiment. Dioxin analyses of these samples would provide a means of validating the simulations. Options for obtaining these analyses are being explored.

Soil ingestion by grazing cattle is an important parameter in assessing the transport of dioxins and other persistent contaminants from the environment to beef. The literature on soil ingestion by grazing animals was reviewed and reinterpreted. Studies of soil ingestion over a full grazing season have typically assumed single values for dry matter intake and digestibility for the full season. These assumptions overestimate soil ingestion when available forage is sparse and digestibility declines late in the grazing season. A sensitivity analysis of soil ingestion data indicates that values used for soil ingestion in typical risk assessments can be reduced by at least 50%.

Close interaction with the FSIS-EPA meat sampling project and the ARS-Fargo metabolic project will be maintained. The results of these projects will be used to validate or modify the models.

PUBLICATIONS:

Fries, G.F. 1994. The sensitivity of measurements of soil ingestion by livestock to assumptions concerning forage intake and digestibility. J. Anim. Sci. 72(Suppl. 1):275. (Abstract).

Fries, G.F. 1994. An overview of the significance of animal food products as potential pathways of human exposure to dioxins. J. Anim. Sci. 72(Suppl. 1):320. (Abstract).

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METHODS TO DETERMINE THE TEMPERATURE TO WHICH PRODUCT HAS BEEN COOKED

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CRIS number: 6612-42000-016
FSIS #: I-5
Completion date: June 6,1996

OBJECTIVE A:

Evaluate the potential for use of residual glutamic-oxaloacetic transaminase (GOT) activity in thermally processed beef and poultry as an indicator of end-point temperatures (EPTs).

PROGRESS A:

In previous research, we showed that GOT activity in beef semimembranosus adductor tissue was eliminated at processing temperatures of approximately 80°C, and that measurement of this enzyme activity had potential for use in determining EPTs in meat products cooked to 70 - 80°C. Continued refinement of analytical procedures for analysis of GOT activity in cooked beef and poultry meats has shown that these analytics are applicable for determining processing temperature of 79.4°C in beef and for determining processing EPTs of 71.1 - 82.2°C in poultry. These processing temperatures are recommended EPTs for processed beef imported from South America and for processed poultry produced at federally inspected establishments, food handlers and by consumers, respectively. In analysis for GOT activity in beef processed to EPTs of 78.8, 79.4 and 80.0°C, values were significantly different at the 5% probability level. No overlapping of values from replicated analysis was apparent between processing temperatures of 78.8 and 79.9°C indicating the reliability of distinguishing accurately between these processing temperatures with these procedures. Some overlapping of values occurred between processing temperatures of 79.4 and 80°C. This overlap was probably caused by the low levels of activity remaining at these temperatures. However, this is of no consequence since the ability to distinguish between processing temperatures of 78.8 and 79.4°C, the critical temperature for inactivation of the foot and mouth disease virus, was established. GOT activity in chicken and turkey breast and thigh meat was determined at 71.1, 73.8, 76.6 and 82.2°C, which are the temperatures recommended by consensus of FDA and FSIS agencies for federally inspected establishments, food retailers, and consumer produced bone-in and whole chickens. GOT activities at these processing temperatures varied significantly at the 5% probability level by processing temperatures, species and tissue within species. Activities were higher in turkey meats than chicken and in the thigh meats of both species. Several commercially prepared turkey and chicken products were obtained from local sources and analyzed for GOT activity to determine if adequate processing temperatures had been achieved. All samples analyzed showed activity levels lower than those in the chicken and turkey samples that had been cooked to the preceding temperatures, indicating that adequate processing temperatures had

been achieved and that GOT activity in these products could be used to determine adequacy of achieving desired EPTs.

OBJECTIVE B:

Determine if the color of juice expressed from beef that has been cooked to 79.4°C can be used as an indicator of end-point temperature.

PROGRESS B:

The preliminary research that was initiated in 1993 has been continued in that (1) spectrophotometric equipment to determine reflectance, absorbance and tristimulus measurements of expressed juices and the cooked meat has been purchased and analytical procedures for these analyses have been established, (2) a Macbeth SpectraLight II has been purchased and installed for use as a standardized light source for subjective evaluation of samples, and (3) preliminary research has been conducted on procedures for preparation and evaluation of samples. No reportable results have been obtained as of this date.

OBJECTIVE C:

Caloric input and lipid migration in heat-processed muscle foods.

PROGRESS C:

Catalase has been used previously as a potential indicator of adequate heating of chicken meat. However, little information regarding the effects of heating rates on catalase activity is available. Experiments were conducted involving samples of ground chicken breast meat that were heated in a water bath at 0 and 2°C above target EPTs of 69, 69.5, 70, 70.5 and 71°C. Catalase activity was determined on 2 g samples in 4 mL of 3% H_2O_2. Formation of air bubbles and floatation, or separation of the solid phase, indicated a positive reaction - precipitation of solids indicated a negative reaction. At EPT of 71°C, no catalase activity was
detected in the samples heated for more than 2 min. The only positive tests were for treatments that required less than 1 min 40 sec heating time at this temperature. At EPT 70°C, the catalase test was negative for treatments of 7 min or longer. These experiments indicate that activity depends not only on EPT, but heating rate as well. Catalase deactivation during heating treatments was affected by the entire cooking process (time and cooking ratio/heating rate). Fat and water content affected cooking time and residual catalase activity but did not affect total process lethality. Total process lethality on residual catalase activity was calculated by integration of the lethality value with cooking time of the process. This procedure offers a potentially simple means for assessing adequacy of heating treatments of chicken breast meat.

OBJECTIVE D:

Conduct a cooperative study among 3 laboratories (ARS, PPMQ, Athens, GA; FSIS Eastern Lab, Athens, Ga; and Dutch Quality House, Gainesville, GA) to determine Acid Phosphatase (ACP) activity in model cooked poultry breast, thigh, and a 50/50 blend of breast and thigh for both marinated and non-marinated meat using the FLUOROPHOS instrument of Advanced Instruments, Inc. Norwood, MA.

PROGRESS D:

A cooperative study involving 3 laboratories (ARS, PPMQ, FSIS, Eastern Lab, and Dutch Quality House) was completed. Commercial non-marinated and marinated poultry breast and thigh were obtained from Dutch Quality House, Gainesville, Ga as raw materials for this study. In addition to the breast and thigh meats, a combination 50/50 blend of both non-marinated and marinated breast and thigh was prepared to simulate a ground patty type product. All sample meats were ground and mixed twice prior to packing 16 g of meat into glass tubes for cooking. All samples were cooked to end-point temperatures (EPT) of °F/68.3°C, 157.5°F/69.7°C, 160°F/71.1°F, 162.5°F/72.5°C, and 165°F/73.9°C. Upon reaching target EPT, sample tubes were placed in an ice/water bath to stop the heating process. Results of the study have shown that the FLUOROPHOS ACP activity could be used as a 15-20 minute analytical method to determine EPT in selected cooked poultry products such as marinated and nonmarinated broiler breast, thigh, and a 50-50 blend of breast and thigh. Statistical analysis shows that the ACP method has a mean -1.1°F (95% confidence limit) below the 160°F FSIS regulation for the tested poultry meat products. The method is now being adapted by the cooperating commercial manufacturer (Advanced Instruments, Inc.) for testing by two commercial processors as a quality assurance HACCP procedure.

OBJECTIVE E:

Evaluate factors that may effect estimation of the end-point temperature (EPT) in product filtrates when using the coagulation test.

PROGRESS E:

Exploratory studies involving factors that may alter the "Coagulation Temperature" of filtrates obtained from extracts of various types of meat and poultry products were conducted. Products included 15 samples of turkey ham roll (5 temperatures x 3 replications) obtained from Michigan State University which had been heat processed to 152.5, 155, 157.5, 160, and 165°F, respectively; six (6) different types of frankfurters (all chicken, all beef, all turkey and mixtures of beef, pork, turkey or chicken, plus one set of frankfurters containing hydrolyzed vegetable protein (HVP), modified starch and whey protein. Cores of links and whole links were analyzed, both as non-frozen and frozen (2 weeks at -10° F). Also, the "Coagulation Test" was conducted on model systems of ground beef, turkey breast, turkey leg, pork loin

and chicken breast heated to EPTs of 150, 155, 160, and 165°F. The coagulation test was also conducted on meats containing 2% NaCl and 0.5% carrageenan heat processed to 150°F and held at 150 for 30 min. Temperature data collected included the temperature at which the filtrate became hazy and at the "first onset of cloudiness" for the muscle tissue without added ingredients; and hazy, cloudy and the presence of a coagulum or protein particles for those muscle tissues containing 2% NaCl and 0.5% carrageenan. A Brinkmann 800 fiber optics dip probe was used to determine changes in absorbance values as samples of the filtrate were removed at the temperature when the filtrate became hazy, cloudy and at the appearance of a coagulum or protein particles. There was fairly good agreement between coagulation temperature and actual EPT for the turkey ham rolls. Coagulation temperature of frankfurters appeared to be dependent on meat type. Cloudiness appeared in the all chicken, all beef, all turkey and mixture of turkey, pork and beef frankfurters, but not in the mixture of chicken, pork and beef, and beef with HVP, modified starch and whey protein frankfurters. Even though all filtrates were heated to 170°F, no coagulum or protein particles appeared. Using the criteria of "first onset of cloudiness" the results showed considerable differences (9-20°F) between coagulation temperature and actual EPT of the sample.

As expected, as EPT of sample increased, the coagulation temperature of the filtrate increased. In general, based on the fiber optics dip probe data, it appears that very little denaturation of proteins (cloudiness) occurs in the filtrates until they reach a temperature of 130 -140°F. In conclusion, the results suggest that some consideration should be given to the use of the criteria of "very cloudy" or to the "presence or absence of a coagulum or protein particles" as an indicator of adequacy of doneness of the product.

PUBLICATIONS:

Senter, S.D., Town send, W.E. and Searcy, G.K. 1994. Variability in residual myoglobin content and glutamic-oxaloacetic transaminase (GOT) activity in cooked bovine semimembranosus tissue as related to temperature of cooking media above end-point temperature and sample size. J. Food Prot. 57: 502-504.

Senter, S.D., Searcy, G.K. and Wilson, R.L. Residual glutamic- oxaloacetic transaminase (GOT) activity in thermally processed poultry and poultry products as an indicator of end-point temperatures. J. Sci. Food and Agric. (In review).

Searcy, G.K., Senter, S.D. and Wilson, R.L. Glutamic-oxaloacetic transaminase (GOT) activity--A potential end-point temperature indicator for imported cooked beef logs. J. Food Prot. (In review).

Senter, S.D., Searcy, G.K. and Townsend, W.E. 1994. Residual GOT activity in chicken breast and thigh meat as an indicator of end-point temperatures. Poultry Sci. 73 (Supplement 1):161. (Abstract #S122).

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Searcy, G.K. and Senter, S.D. 1994. Residual glutamic-oxaloacetic transaminase (GOT) activity in thermally processed poultry and poultry products as an indicator of end-point temperatures. Poultry Sci. 73 (Supplement 1):65. (Abstract #193).

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LIPID OXIDATION PRODUCTS IN FRYING OILS AND FOODS AS POTENTIAL HEALTH HAZARDS

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CRIS #: 5325-42000-014
FSIS #: I-93-3
Completion date December 25, 1996

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OBJECTIVE A:

To identify oxidative and other degradative products, in frying oils and in fried foods, formed in large scale commercial meat and poultry processing operations and during subsequent storage.

PROGRESS A:

Volatile constituents from used frying oils (obtained from various West coast food processing plants) were studied using capillary gas chromatography, combined capillary gas chromatography/mass spectrometry (GC/MS) and combined capillary gas chromatography/Fourier transform infrared spectrometry (GC/FTIR) using headspace sampling and simultaneous distillation-extraction as the sample preparation methods. Over 120 constituents have been identified. Since the determination of frying oil lifetime is one of the goals of this project the identification of marker compounds which increase with oil use would be desirable as a useful index of oil quality. A series of oxo-aldehydes were synthesized and 4-oxohexanal, 4-oxooctanal, 4-oxononanal and 4-oxodecanal were identified as constituents of used frying oil for the first time. Oxo-aldehydes are possible precursors of alkylfurans which are reported to inhibit chemically induced carcinogenesis in mice. A preliminary model thermal degradation study with 4-oxononanal refluxed in hexane for 40 days revealed the formation of the following reaction products: 3-octanone, 2-hexanone, 2-hexanol, 1-methylcyclopentanol, 3-hexanol, 3-hexanone and 2-pentylfuran. Further thermal degradation studies with oxo-aldehydes are in progress and will clarify the role in the production of alkylfurans.

Objectives of planned research:

B. To carry out quantitative analysis of these degradation products to give some idea of their concentration in foods.

C. Evaluate the degree of health hazard of the products identified from known literature information and cooperative tests.

D. Develop practical rapid methods for determining when frying oil should be replaced.

PUBLICATIONS:

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NITROSAMINES IN HAMS PROCESSED WITH ELASTIC RUBBER NETTINGS

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FSIS #: I-92-1
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OBJECTIVE A:

Develop Supercritical Fluid Extraction methodology for nitrosamines of regulatory interest.

PROGRESS A:

Research on the development of a supercritical fluid extraction method (SFE) for nitrosamines, especially N-nitrosodibenzylamine (NDBzA), in hams processed in elastic rubber nettings was completed. Twenty one samples from the outer surface, ranging from N.D. to 157.3 ppb NDBzA, were analyzed by this method and by a solid phase extraction (SPE) method. The results from the two methods were found to be statistically equivalent. The S.D. of the SFE method was 1.7 ppb NDBzA (C.V. 2.7%) compared to 2.2 ppb with a C.V of 3.5% for the SPE method. It is estimated that 20 samples can be analyzed per day by the SFE method compared to a maximum of 8-10 for the SPE method. Considerably less dichloromethane (DCM) is also required. SFE is currently being carried out on N-nitrosopyrrolidine in fried bacon.

OBJECTIVE B:

Determine mechanisms for nitrosamine formation in netted hams.

PROGRESS B:

Zinc dithiocarbamates are used as vulcanization accelerators in the formulation of rubber. They are also thought to be the source of NDBzA in hams exposed to the rubber in the elastic nettings. Studies on the thermal decomposition of purified zinc dibenzyldithiocarbamate (Zn DBzDTC), by a variety of thermal and spectroscopic techniques, were completed. The Differential Scanning Calorimetry (DSC) and Thermogravimetric Analysis (TGA) showed that 326° C was the decomposition temperature of Zn DBzDTC. TGA-FTIR indicated that the dominant decomposition product was CS_2; there was no evidence of dibenzylamine (DBzA). The temperature of the interface could not be heated sufficiently high to pass this compound into the light pipe. Zn DBzDTC was injected into a GC-MS, whose injection port was heated from 200° to 300°C in 20°C increments. The amount of dibenzylamine (DBzA) increased with temperature. Even considering that rubber is formulated with compounds that facilitate accelerator

decomposition, the high temperature of decomposition suggests that undecomposed accelerator is still present in the rubber netting, even after smokehouse processing. It also suggests that the amount of free DBzA, present as a contaminant in the crude commercial accelerator, may play a more important role in nitrosamine formation than first thought. Additional research in this area will not continue, except in the form of assessment of the nitrosation potential of the rubber, rubber nettings or ham product.

OBJECTIVE C:

Effect of processing on nitrosamine formation.

PROGRESS C:

Since the last annual FSIS/ARS Food Safety Research Planning Workshop, most of the effort has been placed on analyzing hams, obtained from FSIS, for nitrosamines. In the initial survey, 59 ham surface samples were obtained from 55 different processors and analyzed in duplicate. Of these, only 3 samples contained N-nitrosodibutylamine (NDBA). One sample had 33.4 ppb NDBA with 4.0 ppb NDBzA and 2 contained 3.8 and 4.2 ppb NDBA with 107.7 and 25.5 ppb NDBzA, respectively. Seven contained no detectable nitrosamines. However, of the remaining samples, 4.0 to 512.2 ppb NDBzA was detected, with 1/3 over 100 ppb. Additional hams were obtained from the same processors whose samples yielded high results in the initial survey. The repeats tended to give variable results, but the results from 3 processors were consistently high; 512.2 and 746.9, 370.6 and 291.0, 486.8 and 550.3 ppb NDBzA. All of the samples containing over 100 ppb NDBzA were confirmed by mass spectrometry. These findings, and a description of the nettings used for processing, were reported to FSIS on a frequent basis. Current work is focussing on the analysis of the nettings that were supplied with the ham samples. Additional ham samples have been requested from processors that gave no detectable nitrosamines. This may help explain and identify the reason for this occurrence.

In another study, the statistical analysis of the NDBzA data ($n=33$) from the outer surface of the ham versus a 1/4 in. thick center cut slice was performed to determine if a correlation was apparent between the two nitrosamine values. Only hams having approximately the same diameter (5 1/4 in.) were used in this study in order to eliminate any effect due to differences in area. The NDBzA values for the outer surface covered a range from 11.5 to 805.1 ppb. A highly significant correlation ($p<0.01$) between the outer and slice values was found. From the predictive equation developed, approximately 14% of the outer NDBzA level will be found in the slice. Or, expressing it differently, the surface to slice ratio for NDBzA was 7.1:1. There are no immediate plans to continue in this area of investigation.

OBJECTIVE D:

Develop methods to reduce/eliminate nitrosamine formation.

PROGRESS D:

Earlier studies on nitrosamine and amine penetration into hams showed that a significant amount of dibenzylamine could be present at a depth of 1 1/2 inches from the ham surface. Addition of nitrite to ham surface samples also confirmed that it had the potential to form additional nitrosamine. This suggested that ingoing nitrite might be an important factor in determining how much NDBzA was formed. Hams were processed in nettings from the same batch; 12 were processed with 200 ppm and 12 with 100 ppm sodium nitrite. The NDBzA content was found to be 1/3 lower with the 100 ppm nitrite than the 200 ppm processed samples. While reduction of ingoing nitrite may be one way to reduce the nitrosamine content in netted hams, further investigations are needed before pursuing this approach.

A study is currently underway to examine the effect of leaving the nettings on hams stored post-processing at refrigeration temperature (-1° to +1°C) for up to 12 weeks. Some ham producers leave the nettings on the hams for esthetic reasons. They can also be kept refrigerated for a long period of time. Samples were taken for analysis at 4 week intervals. Preliminary results show that the NDBzA values gradually increase on the outer surface, then start to decrease; whereas, the NDBzA levels in the nettings themselves generally decrease over the 12 week storage. The increase, then decrease of NDBzA on the outer surface of the ham may be due to penetration of pre-formed NDBzA from the netting into the ham. Additional storage experiments are underway to determine if this is the case. Future experiments, using other approaches, are planned to reduce the nitrosamine content of hams processed in elastic rubber nettings.

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO ANALYSIS OF FIRE-EXPOSED MEAT SURFACES

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OBJECTIVE A:

Utilize supercritical fluid extraction to obtain volatile and semivolatile compounds from fire-exposed meat products.

PROGRESS A:

This new project, developed in collaboration with the Midwest FSIS Laboratory, has been reduced to practice during this past reporting period. SFE, both off-line and on-line, has been shown to be applicable to the analysis of volatile or semivolatile compounds in fire-damaged meat products. The basis for this approach, which includes both selective SFE as well as SF-desorption of volatiles/semivolatiles from Tenax trapping resin, was initially developed for characterizing volatiles/semivolatiles in abused and stored vegetable oils. A similar approach was also utilized for the SFE of volatiles/semivolatiles from meat matrices, including marker components which would indicate that the samples had been exposed to fire. Successful characterization of volatile/semivolatile components from the above vegetable oils was initially accomplished by collection on Tenax, followed by desorption with SC-CO_2, by utilizing SFE in-line, with a GC/MS system. The solvation/desorption power of SC-CO_2 was sufficient to allow the analysis of components having carbon numbers in excess of C20. Results from SFE/GC/MS experiments also indicated the absence of certain volatile compounds found in headspace/GC/MS approach which uses thermal desorption to remove the adsorbed compounds from the resin. The headspace conditions apparently can degrade some of the unsaturated lipid compounds due to the high desorption temperatures that are required. Desorbing the analytes with SC-CO_2 prevents the formation of these volatile components due to the benign nature of the SF-desorption process (inert CO_2 atmosphere, extraction temperature of 50°C).

The above approach has been put to good use in the on-line SFE analysis of lipids in fire-exposed meat samples. In most of the samples examined to date, elevated levels of aldehydes, such as hexanal or nonanal, have been found to be indicative of meat exposure to fire. These elevated aldehyde levels have been found to be higher in meat samples taken from a cave fire in which commercial products were exposed to smoke and heat. Likewise, SFE has also showed the capability of extracting heterocylic amines from meat. Such compounds have been found to be associated with heat-induced reactions between protein and carbohydrate moieties in foodstuffs.

OBJECTIVE B:

Design and test on- and off-line SFE with appropriate analytical methodology for the identification and quantitation of extracted chemicals.

PROGRESS B:

Direct SFE of meats can also be accomplished at the low extraction temperatures noted above in Objective A with SC-CO_2. Using these conditions, an on-line SFE procedure was developed, whereby SFE was coupled with a GC/MS/DS system, for volatiles/ semivolatiles analysis. Using extraction conditions of 60°C and 100 atm, permitted direct extraction of the volatile/semivolatile compounds while minimizing the carryover of lipids into the GC/MS system.

A new SFE/GC/MS/DS system was constructed for these studies using a Incos 50 MS/DS, in conjunction with a Varian 3400 GC and a Suprex Prepmaster SFE unit. Extracts were transferred into the GC/MS system using a specially constructed heated transfer line, terminated by a frit restrictor, which was inserted into the injector of the gas chromatograph. Cryotrapping of the volatiles was affected before initiating the programmed temperature gas chromatographic run. Compounds were quantified using the single ion mode produced by electron impact, in conjunction with calibration standards and deuterated internal standards of naphthalene. Approximately 0.5 gram meat samples were taken for analysis.

To assist in the optimization of trapping volatiles/semivolatiles on sorbent resins after SFE, a study of compound breakthrough volumes from Tenax-TA and XAD-2 resins was made using an inverse GC technique. Current breakthrough volumes on Tenax are largely determined for helium as a carrier gas, which is not utilized in SFE. Precise specific retention volume measurements were made on an NCAUR-designed physicochemical gas chromatograph. Retention data was accumulated for over 25 compounds for 6 classes of compounds in both helium and carbon dioxide carrier gases. Retention volumes were also measured at a least three different temperatures and the van't Hoff relationship used to obtain extrapolated retention volumes at 4 different trapping temperatures. The retention (breakthrough) volumes for most of the above solutes were found to be considerably lower in CO_2 than in helium carrier gas. This confirms that estimates of breakthrough volume based on measurements conducted in helium carrier gas will lead to a considerable error in estimating the retention capacity of sorbents used for trapping volatiles from SFE. A useful correlation was developed for relating the specific retention volume (breakthrough volume) to the boiling point of the extracted analyte, resulting in a predictive scheme for estimating the breakthrough volumes for unknown analytes without having to resort to experimental measurement.

OBJECTIVE C:

Identify and quantify marker compounds associated with meat exposure to fire or chemicals in cooperation with FSIS.

PROGRESS C:

Visits to the FSIS Midwest Laboratory, coupled with a return visit from Carolyn Henry and her staff to NCAUR, resulted in the transfer of specific meat samples from the Americold cave fire in Kansas City. These samples have been extracted by SFE and directly introduced into the injection port of the above GC, as described in Objective B. Major components that are associated with fire exposure, have been identified with the aid of the MS/DS. Most of these are PAHs and heterocyclic nitrogen compounds. The presence of naphthalene was confirmed in all of the fire-exposed meat samples by the single ion monitoring technique. In some cases, small amounts of naphthalene were also found in the control samples of the commercially-smoked meats. The amounts of naphthalene found in meats such as ham, smoked chicken, turkey breast, and corn beef, using SFE, tended to be somewhat lower than those recorded by analysts at FSIS's Western Laboratory, using headspace techniques. However NCAUR analysis of control meat samples using SFE showed less naphathalene than found by the FSIS laboratory. Results using both external and internal standardization indicated the presence of similar levels of naphthalene in all of the samples that were run. Hence, it appears that SFE of naphthalene from meat samples, followed by GC/MS identification and quantitation, is a reliable marker of meat exposure to fire.

Additional analysis on the above meat samples have measured the amount of toluene, ethylbenzene, and xylene in five commercial products. The level of these components also appear to be reliable indicators of exposure to fire conditions. We have also, at the request of FSIS's Midwestern Laboratory, examined cooked hamburger and bacon bits for volatiles producing off-flavor notes during a cooking process. No significant differences were noted for the bacon bits samples, however the cooked hamburger patties which produced the off odors, when analyzed by the SFE/GC/MS/DS technique, yielded two new compounds, one which was tentatively identified as 5-hydroxy methylfurfural.

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APPLICATION OF SUPERCRITICAL FLUID TECHNIQUES TO DETERMINATION OF FAT LEVELS IN FOOD PRODUCTS

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CRIS #: 3620-42000-13
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OBJECTIVE A:

Develop supercritical fluid techniques for the determination of fat levels in meat and food products according to NLEA methods.

PROGRESS A:

Much effort has been focused on understanding the many parameters which influence the extraction of fat from meat and other food products. This effort is due in part to understand the differences in the newly mandated NLEA method for "total" fat, which is considerably different and more complex than classical Soxhlet or solvent extraction procedures using a variety of organic solvents. Two of the major objectives of the NLEA fat method are to assure the extraction of "bound" lipid matter and to provide an analyte (fat) specific assay method. It is within this context that we are attempting to insert SFE in place of traditional extraction methodology.

Extractions have been performed on a variety of foods (potato chips, low and high fat cookies, M&M peanut chocolates, and corn chips), in order to study foods that have been prepared with refined oils (low phospholipid content), rather than ill-defined fat-containing products, such as meats. Extractions performed sequentially using SC-CO_2 followed by a SC-CO_2/ ethanol mixture have indicated that little if any additional lipid matter is extracted from the above foods which are processed or compounded with refined fats/oils. This is undoubtedly due to the fact that phospholipids and other minor polar lipid constituents have already been removed from the fats or oils in the commercial refining process.

However, the use of cosolvent/SC-CO_2 mixtures on meat samples produces variable results when gravimetry is used to assess fat levels. SFEs performed on ground turkey patties using several cosolvents and a variety of SFE conditions have shown variable results, in fact, the percentages of fat can be made to vary up to 100% depending on the cosolvent that is used, the hydration state of the meat sample, and the mode of SFE that is utilized. It should be noted that extraction with organic liquid solvents also produces these erratic results, as has been known for sometime. Recent engineering scale extraction studies in our laboratory on soy lecithin have indicated that only specific neat phospholipids can be solubilized in

CO_2/cosolvent mixtures, e.g. SC-CO_2/15 mole % ethanol only solubilizes phosphatidylcholine and phosphatidylethanolamine, leaving behind the more polar phospholipids.

To establish a baseline, several ground turkey samples were submitted to Medallion Laboratories (Minneapolis, MN) for extraction and subsequent analysis according to the new NLEA protocol. Ground turkey samples were also extracted at NCAUR on the same turkey patties incorporating pre-SFE acid hydrolysis of the meat sample to release bound fat. SFEs were performed on NCAUR-built extractors and a commercial Hewlett Packard SFE. Results from the SFEs using gravimetric assay gave variable results due to the lack of an analyte specific assay and inhomogeneity of the turkey samples, when compared with the results from Medallion Labs. For this reason, a study was initiated with the Department of Meat Science at the University of Illinois to produce standard, homogenized meat samples of known fat content.

Considerable effort has been made this past reporting period in implementing the hydrolytic fat method using FAME analysis as provided to us by Steven House of General Mills, Inc. This quantitative assay was more complex than originally thought, but if carefully followed consistently yields accurate and reproducible results. NLEA-type fat analyses were eventually performed at NCAUR on a standard ground beef sample provided by the University of Illinois at NCAUR, using SFE, and compared with values generated by Medallion Labs using an identical procedure, but conventional solvent extraction to isolate the fat. Results from home-built, as well as one commercial extractor, were in good agreement with the Medallion data for total, saturated, and monounsaturated fat. This was confirmed using several packets of the homogenized ground beef as well as subsamples on a individual one pound packet. Studies on this sample were extended using the Hewlett Packard 7680T extractor operating at 5700 psi, and shown to yield equivalent results to data obtained at 10,000 psi extraction pressure. Studies are continuing on this and similar samples using other commercial extractors.

OBJECTIVE B:

Conduct collaborative study to show equivalence of SFE-based method to NLEA and similar protocols for fat analysis.

PROGRESS B:

The complexities of the NLEA total fat method noted in Objective A have delayed the initiation of the collaborative study. However, preparation of standard meat samples for the collaborative study have been completed during this reporting period. As noted above, an initial lot of ground beef has been prepared by the Department of Meat Science at the University of Illinois from beef trimmings. The beef was ground through a 13 mm diameter plate, mixed in a ribbon mixer, and then reground through a 3 mm diameter plate. The ground product was further homogenized in a bowl cutter until fat was no longer

distinguishable in the final product. This product was inserted into vacuum bags, vacuum packaged, and frozen at -20°C.

Evaluation of the fat level by Medallion Labs using NLEA methodology indicated that the samples were highly homogenized. As noted above, SFE of the same meat samples produced fat levels equivalent to those obtained using the NLEA protocol. Based on these results, additional samples were prepared at the University of Illinois consisting of 3 fat levels in ground beef, 2 fat levels in processed hams, a processed bacon standard, and 3 fat levels in bologna. The ham and bologna were further treated by smoking in an oven. All of the above samples were then homogenized by the procedure described previously for the ground beef sample. Assays are currently being run at Medallion Labs using the NLEA total fat method on triplicate samples of each meat type and corresponding fat levels. Some of these samples will then be extracted at NCAUR via SFE for comparison to the Medallion data. All of the above samples will form the sample pool for the collaborative study.

OBJECTIVE C:

Apply SFC for the analysis of cholesterol and fat soluble vitamins in meat products.

PROGRESS C:

The Hewlett Packard Model G1205A SFC has been utilized for optimizing the chromatographic conditions for the separation of fat soluble vitamins this past reporting period. Several types of columns, isobaric conditions, and cosolvents were utilized to separate the following vitamins: vitamins E, D2, D3, A and K. Multiple column banks (3-5) of packed silica columns proved most effective at 150 bar and 40°C, using 1-5% methanol modifier, for separating the above standard vitamin mixtures. Some initial separations of vitamin E in pharmaceutical preparations showed the possibility of separating and specifically detecting vitamins in lipid-containing mixtures, using ultraviolet detection.

The possibility of using SEC to separate the vitamin moieties from fat was also explored. Using Jordi Gel size exclusion columns, in conjunction with the HP SFC, permitted the fractionation of most of the above vitamins away from fat, although individual vitamins coeluted after exclusion of the fat from the resin matrix in the column. Hence, although this method might not prove applicable for individual determination of the vitamins, the approach could be used to isolate the vitamins, fat-free, for further assay.

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REFERENCE MATERIALS AND METHODOLOGY FOR NUTRIENT ANALYSIS

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CRIS#: 1235-52000-026
1235-52000-028
FSIS #: 1-93-1
Completion Date 1996

OBJECTIVE A:

Conduct research to increase knowledge and technology in matrix/analyte stability and homogeneity and to develop highly accurate methodology applicable to the production of certified reference materials for foods.

PROGRESS A:

In collaboration with the National Institute of Standards and Technology (NIST), a definitive analytical method was developed for selected fatty acids in foods employing isotope dilution/gas chromatography/mass spectrometry (ID/GC/MS). This technique was applied to the determination of fatty acids and cholesterol in a frozen composite human diet Standard Reference Material (SRM). High precision GC analysis was used to confirm ID/GC/MS results.

A project to study the effect of several long-term storage temperatures for fresh-frozen and freeze-dried diet materials on lipid components is on-going. Research studies of reference material homogeneity through generation of sampling constant data for organic constituents are continuing.

OBJECTIVE B:

Establish a national program to provide analytical food reference materials to meet requirements for the Nutritional Labeling and Education Act for FSIS, FDA and the private sector.

PROGRESS B:

Infrastructure for a multicomponent approach to provide food based reference materials for the U.S. is being developed. A Technical Division on Reference Materials has been established within AOAC International. An ongoing project of the Division is to establish a database of recommended reference materials for use with specific AOAC International methods. A Task Force has also been established to develop guidelines for use of multi-lab data in characterizing reference materials. An approach for development of food reference materials based on protein, carbohydrate and fat content has been developed. Nine matrices

based on proportional composition of protein, carbohydrate and fat were identified as representative of all foods.

OBJECTIVE C:

Develop appropriate analytical field methodology for the measurement of nutrients in foods as part of nutritional labeling activity.

PROGRESS C:

Procedures for the extraction, saponification and separation of food tocopherols and tocotrienols employing an internal standard were developed. Tocol was selected as the internal standard after demonstration of adequate recoveries in extraction and saponification steps. Normal phase high performance liquid chromatographic separation with fluorescent detection is used for quantification of each tocopherol and tocotrienol component. The overall procedure is sensitive and yields clean chromatographic baselines.

PUBLICATIONS:

None

NIR SPECTROMETRY TO MEASURE NUTRIENTS

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CRIS #: 6612-44000-013
FSIS #: I-94-3
Completion date

OBJECTIVE:

To study the usefulness on NIR technology in nutrient analysis of meat and poultry products for nutrition labeling and other regulatory samples and assess its ability to reduce the amount of hazardous waste from current laboratory methods.

PROGRESS:

Sample preparation and sub-sampling for analysis:

The influence of sample preparation, sampling, and number of scan locations averaged on near infrared transmittance (NIT) analysis of fat and moisture in ground beef (GB) were determined. Thirty-five GB samples, ranging from 12 to 36% fat and 51 to 67% moisture were used. Samples were ground by two procedures: 1) three times through a 2.0 mm hole diameter plate and 2) emulsified for 50 s in a vertical cutter (V/C) mixer. Mean spectral values were obtained from 5 to 30 scan locations in increments of 5 nm from 850 to 1050 nm on 4 sub-samples (for each meat sample). Observed fat and moisture values were not different ($P > 0.05$) for sub-samples or number of scan locations. Averaged for both sample preparations, the standard error of the means (SEM) due to sub-samples for predicted fat and moisture were 0.48 and 0.36, respectively. Variations in SEM values across number of scan locations were minimal for both plate and V/C mixer preparations. With sample preparations as described, we concluded that one sub-sample with five average scans provides accurate and precise analysis of fat and moisture.

Sample temperature and analysis:

The influence of sample temperature on NIT analysis of fat and moisture in ground beef (GB) were determined. Twenty five GB samples, ranging from 15 to 35% fat and 50 to 64% moisture. Samples were chilled for 24 h at 5°C, 0°C, -4°C, and -12°C. After chilling treatment, samples were scanned and predicted for fat and moisture concentration. At -4 and -12°C treatments, the 960 nm water absorption band shifted 20 nm to a higher wavelength. As a result, if a fat and moisture calibration equation without terms for temperature were used, the predicted moisture values will be significantly lower than the laboratory reference data. Work is continuing on development of fat and moisture NIT equations that are not sensitive to sample temperature.

PUBLICATIONS:

Windham, W. R., F. E. Barton, II and K.C. Lawrence. 1994. Influence of sample preparation and sampling on NIT analysis of fat and moisture in ground beef. Proc. 6th Intern. Conf. on Near Infrared Spectroscopy. Lorne, VIC., Australia, (In press).

DETECTION OF EXTRANEOUS MATERIALS BY X-RAY

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FSIS #: I-90-4
Completion Date: December 31, 1994

OBJECTIVE:

Develop specifications for line-scanning x-ray systems to be used for screening of organ samples for extraneous materials by the three FSIS regional laboratories.

PROGRESS:

Six hundred processed meat samples received at the FSIS St. Louis Midwest laboratory have been x-rayed by digital scanning equipment upon receipt. Two hundred and forty of these were spiked [blind to the analyst] with assorted targets to establish recognition rate. Experimental design spanned the five variables believed to affect recognition: target thickness [1-10 cm], target x-ray texture [smooth, textured], spike thickness [1-4 mm] spike shape [sharp, rounded], and spike composition [wood, glass, bone]. Analysis still needs to be completed, but preliminary results indicate that bone is almost always recognized, glass under favorable conditions, while wood is almost never seen. From this data recognition of other inclusions, such as plastic and metal, can be predicted. While the incoming sample load is limited, it appears that, absent a processing problem, only bone inclusions are common.

PUBLICATIONS:

Tollner, E.W., H.A. Affeldt, Jr., G.K. Brown, P.K. Chen, W.R. Forbus, Jr., N. Galili, C.A. Haugh, A. Notea, Y. Sarig, T. Schatzki, J. Smulevich, and B. Zion. 1994. Nondestructive Detection of Interior Voids, Foreign Inclusions and Pests in "Nondestructive Technologies for Quality Evaluation of Fruits and Vegetables", Soc. Agric. Engin., St. Joseph, MI.

ARS FOOD SAFETY RESEARACH ADDRESSING FSIS NEEDS

Subject Area and Laboratory Location Index

II. Pathogen control during slaughtering and processing

III. Post-slaughter pathogen control

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